GLYCOGEN-RICH CELLS IN EARLY TOOTH FORMATION

A TEM AND *IN VITRO* STUDY

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SUMMARY

It is still uncertain if neural crest cells initiate tooth
development in mammals, since current information is extrapolated from
studies on avian and amphibian embryos. This investigation was undertaken
to examine the putative role of neural crest ectomesenchyme in mammalian
tooth formation; making use of glycogen-content as the criterion for its
identification.

Mouse embryos; 12½, 13½ and 14½ days in gestation were sacrificed
and the heads serially sectioned to determine the first sign of tooth
development in the maxilla. The tooth bearing areas, of the maxillary
first molars, were then subjected to ultrastructural study under the
transmission electron microscope. Following this, the tooth bearing areas
from the same sites were excised and cultivated as organ cultures for
varying periods of time.

The first sign of tooth development was seen in 12½ day old embryos,
in the form of a thickened dental lamina. At 13½ days, a bud-shaped structure
was seen to evaginate into the underlying mesenchyme. Under the TEM, the
most salient observation was the sudden and dramatic appearance of cells
rich in glycogen within the dental mesenchyme of 13½ day old embryos only.
These cells possessed features suggestive of migratory activity and were
significantly related to a rich extracellular matrix. In 12½ day old tissues,
the mesenchyme was free of glycogen-rich cells except for the perivascular
areas distant to the dental lamina. When the presumptive dental tissues
were cultured, only explants taken from 13½ day old embryos produced teeth;
even to the extent of enamel and dentine secretion. Explants taken from
12½ day old embryos consistently failed to produce teeth; only stratified

*epithelia with disoriented mesenchyme and occasionally, bone and
cartilage.*

*A definite cause and effect relationship was established between
ability to form teeth in culture and the presence of glycogen-rich cells
within the explants. A case is therefore presented to suggest that the
glycogen-rich cells are of neural crest origin. The implications of this
conclusion to tooth development generally, were also discussed.*

SIGNED STATEMENT

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.

Seong-Seng TAN

ACKNOWLEDGEMENTS
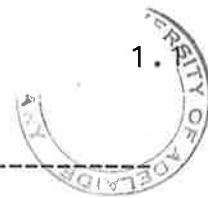
Chapter 1. INTRODUCTION

The neural crest is a transitory structure which appears early in the vertebrate embryo. Its cells have the striking property of migrating to widely separated positions in the embryo and in their final locations, differentiate into a variety of physiologically unique cell-types(see reviews HORSTADIUS, 1950; WESTON, 1970). The exact developmental repertoire of this prolific structure has never been fully discovered and after more than a hundred years of research, its position in zoology remains an enigma.

A survey of the literature will reveal more questions than answers. While it is almost certain that neural crest cells reach their migratory end-points via well-defined pathways, little is known about the mechanisms of motility and the factors affecting their directionality. It is also unclear as to what triggers the initiation or arrest of migration. The question of pluripotentiality looms unsettled despite the elegant experiments of LE DOUARIN(see review, 1980) and her colleagues. Most important of all, the nature of the inductive influences that affect neural crest differentiation is poorly understood.

Meanwhile, the dental literature is replete with statements which imply without qualification the neural crest origin of dentine and periodontium in humans(SLAVKIN, 1974; JOHNSTON, 1976; OSBORN & TEN CATE, 1976). Such extrapolations become even more tenuous when it is realised that precise studies on the origin, migration and differentiation of neural crest cells have so far been performed only on avian and amphibian embryos. Although there is general agreement that mammalian neural crest

behave in a manner essentially similar to those of lower vertebrates, great caution must be exercised when experimental evidence from non-mammalian embryos is relied upon.

To date, there are no published accounts of direct experimentation with the neural crest and its role in mammalian tooth formation. This is understandably due to the relative inaccessibility of the mammalian embryo to intervention during the post-implantation stages. The other technical difficulty is the lack of a suitable cell marker. The above limitations have not discouraged indirect experimentation; histochemistry has been employed to trace the migration of neural crest cells in rodents (DALCQ, 1953; MILAIRE, 1953; POURTOIS, 1961). These workers have, significantly, reported streams of neural crest cells directed towards the craniofacial regions; identification was easy because of their high RNA, alkaline phosphatase and glycogen content.

If glycogen is a distinctive feature of neural crest cells, then one should expect to see a migratory population of cells, rich in glycogen, arriving into the presumptive dental anlagen. Features indicative of cellular motility would be expected on ultrastructural examination of these cells. A corroboration of glycogen content together with evidence of cellular locomotion(at the appropriate developmental age) would be the first step toward our understanding of neural crest participation in mammalian odontogenesis. The contribution of the glycogen-rich cells to tooth formation could also be assessed by suitably designed organ culture experiments. However, the reader should be forewarned that this study is designed to provide circumstantial evidence only; utilizing criteria previously defined by other investigators(MILAIRE, 1959; POURTOIS, 1961). For a full confirmation, direct manipulation of the neural crest is warranted.

Chapter 2. AIMS OF THE INVESTIGATION
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1) To obtain evidence of neural crest ectomesenchyme migration
   into the presumptive dental tissues.

2) To test the role of neural crest cells in tooth formation by
   in vitro experimentation.

3) To encourage hard tissue(enamel and dentine) formation in
   explants grown at the dental lamina stage.

A HISTORICAL PERSPECTIVE

According to HORSTADIUS(1950), the first reference to the neural crest(NC) was made by HIS(1868) who described its contributions to cranial and spinal ganglia in chick embryos. KATSCHENKO(1888) subsequently suggested the NC origin of head mesenchyme but direct evidence was only produced later by PLATT(1896). She noted that the ectodermal cells of both NC and placodes(in Necturus) gave rise in addition to cranial and sensory ganglia, connective tissue, cartilage and dental pulp. Hence the term 'mesectoderm' was coined to embrace all ectodermal cells capable of producing skeletal and connective tissues.

THE GERM LAYER THEORY

At first, many scientists denied the possibility of mesenchyme arising from ectoderm because of the belief in the germ layer theory. This theory claims two things:

a) that in normal development from the egg, the materials out of which the primary organ systems arise are arranged simply in layers; the ectoderm, endoderm and mesoderm.

b) that homologous structures in different types of animals are consistently found to arise from corresponding layers.

The concept of homology was often found to be in difficulties. For example, in the Metazoa, muscles are generally regarded to be derived from mesoderm but in certain Crustacea, some muscles are derived from

ectoderm(DE BEER, 1947).

The conclusions of PLATT(1896, 1898) were met with severe criticism from adherents of the germ layer theory. So effective was their opposition that for the next 20 years, little was published regarding the role of the NC and placodes in embryology. However, it didn't take long for the neural crest to become the target of intensive enquiry again. The migratory behaviour of NC cells sets them out as the most motile embryonic structure and was soon the favourite tissue utilized by workers attempting to elucidate the intricate mechanisms of cellular motility. The versatility of the NC, expressed by its wide ranging embryonic cell types, has intrigued scientists for over a hundred years now; and in spite of real gains in our understanding, it remains a baffling structure.

The mystery surrounding the neural crest is brought to a head when one proceeds to examine evidence for its putative role in tooth formation. At first, descriptions were made by anatomists who studying the derivatives of NC cells in amphibia, merely noted their presence in dental pulp(PLATT, 1898; RAVEN, 1935). This theme was seized upon by others who, lacking a real understanding of NC behaviour in other parts of the embryo, proceeded to describe its contribution to mammalian teeth and in not a single instance was concrete evidence produced. The role of the NC in odontogenesis cannot be examined in isolation, a frequent mistake in the past. Any study that has for its end objective the elucidation of a NC derivative will require a comprehensive knowledge of NC ontogeny(and phylogeny) before the experimental date can be interpreted intelligently. Such an approach has been elected for this stydy, justifying the comprehensive review of the literature.

THE NEURAL CREST IN GENERAL

It will be appropriate to preface this review by saying that the information set out below is basically applicable to avian and amphibian embryos only. While there is no reason to assume that the mammalian neural crest behave in a dissimilar fashion, little experimental work has been performed to verify this assumption. Significant differences in the pattern of adult structure between mammalian and non-mammalian vertebrates would suggest equivalent variations in NC cell behaviour. These differences will be emphasized in the appropriate parts of the review which initially, is divided into the following subsections.

NEURAL CREST DEVELOPMENT

NEURAL CREST MIGRATION

NEURAL CREST DIFFERENTIATION

NEURAL CREST EXPERIMENTATION

NEURAL CREST DEVELOPMENT

FORMATION

During embryogenesis, the ectoderm of the bilaminar stage embryo provides a source of embryonic mesenchyme in two separate events. The first involves the conversion of the epiblast cells to primary mesenchyme(mesodermal mesenchyme) in the primitive streak resulting in a trilaminar structure(Fig. 1). In the second event, the thickened ectoderm rises up as the neural folds, fusing together in the midline and converting the plate into a tube which sinks beneath the surface ectoderm(Fig. 2). During this process, termed neurulation, certain
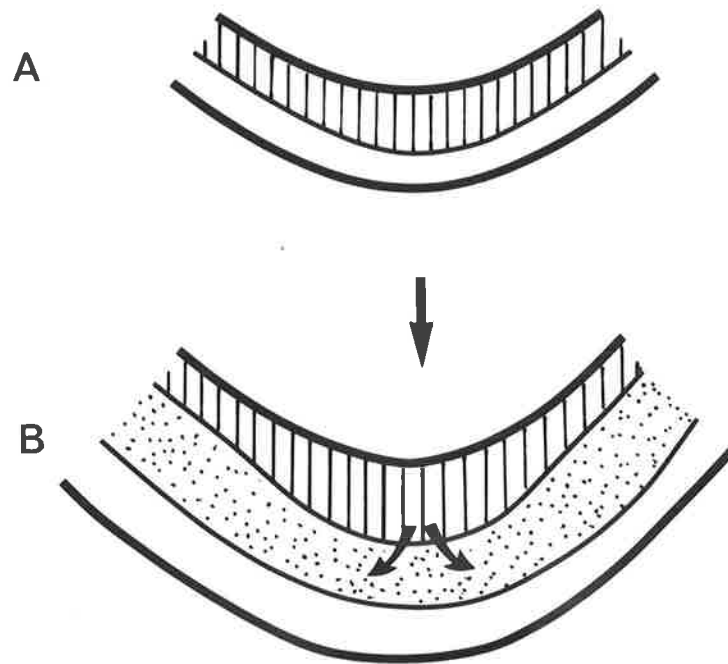
Fig.1 Transverse view of the primitive streak region showing
     the formation of primary mesenchyme(stippled) as a
     result of migration from epiblast cells(striped).

     (A):Bilaminar stage embryo
     (B):Trilaminar stage embryo
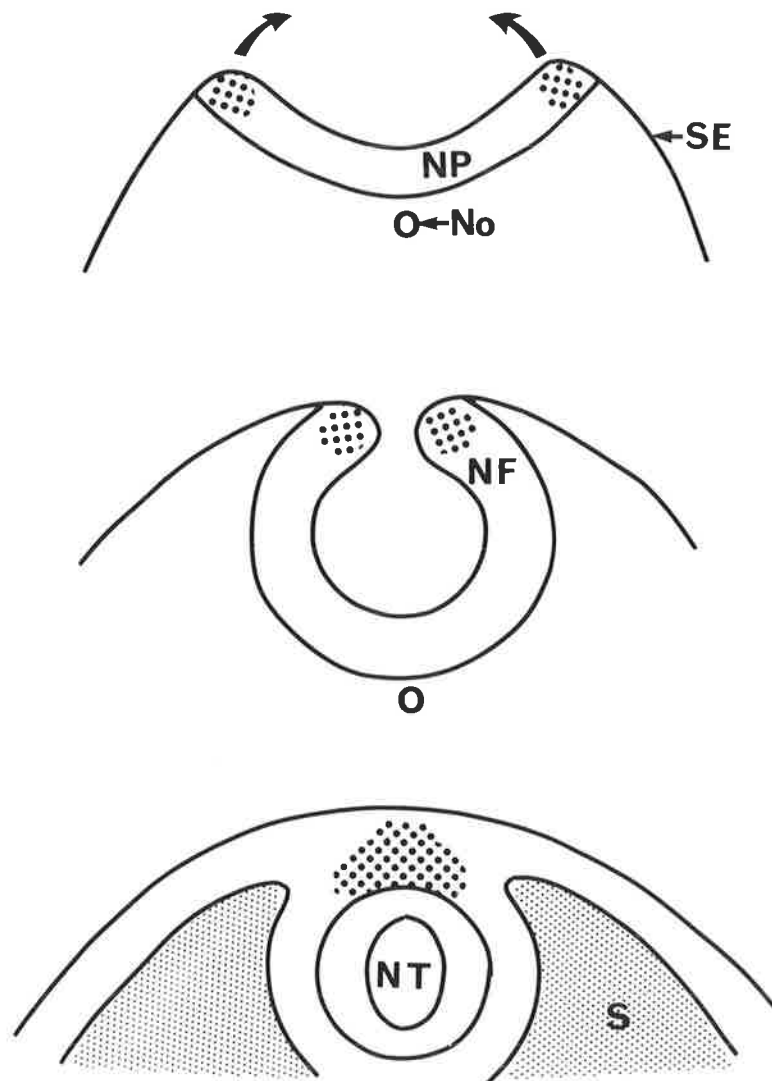
Fig.2 Neural crest cells(large dots) originate from the most
      lateral part of the neural plate(NP) and eventually
      come to lie on the dorsal aspect of the neural tube(NT)
      at the completion of neurulation.

      Neural Plate(NP), Neural Fold(NF), Neural Tube(NT),
      Surface Ectoderm(SE), Notochord(No), Somites(S).

      Neural crest cells.

ectodermal cells break away from the lateral margins of the neural
plate and come to lie on the dorsal aspect of the neural tube. These
are the NC cells and are designated ectomesenchyme(or mesectoderm) in
order to emphasize its distinction from mesodermal mesenchyme. Because
they appear at such an early stage of embryonic life and gives rise to
a wide range of tissues, it behaves almost as a fourth germ layer(NEWTH,
1951).

MORPHOLOGICAL CHANGES

NC cells undergo changes in form and shape, reflecting alterations
in their migratory behaviour. In the avian embryo, the neural crest can
be classified as immature, intermediate and mature depending on its
position on the anterior-posterior axis of the neural tube(Bancroft &
Bellairs,1976). This gradient of maturity is associated with the onset
of migration; evidence of NC cell migration being found most frequently
in mature areas and associated with three important features under the
scanning electron microscope(SEM) viz.,

1) re-orientation of the cells in a medio-lateral direction

2) dorso-ventral flattening of the cells

3) reduction in intercellular spaces.

In addition, the cells in the areas of heaviest migration were seen to
be laterally orientated, with their thickest ends lying distal to the
neural tube. These observations were confirmed by TOSNEY(1978) who
defined changes in NC morphology into four phases: appearance, condensation,
early migration and advanced migration. She found that the NC cells
changed from a dorsal projection to elongated and flattened cells
as they prepare to migrate. In addition, they had a tangential orientation
perpendicular to the long axis of the neural tube with numerous filopodia

at the leading edges. There was also a decrease in the intercellular spaces with advancing phases of NC cell migration. In contrast, cells in advanced migration were stellate in form, possessed multiple processes, without obvious orientation and often separated from each other by wide spaces. In the most recent SEM study(STEFFEK, MUJWID & JOHNSTON, 1979), migrating NC cells were described as bipolar in form and attached to other cells by means of filopodia or microspikes.

In the amphibian embryo, SEM studies have revealed that similar morphological changes are associated with the onset of NC cell migration (LOFBERG & AHLFORS, 1978). In particular, the NC cells change from an anterior-posterior orientation to a ventro-lateral position over the neural tube. At the same time, the cells become flattened while filopodia, cone-shaped processes and microvilli are frequently seen at the leading edge of the population.

Morphological changes during NC cell migration *in vitro* have also been reported. MORAN(1978) found that cell movement was performed by angulated cells only, and at the cessation of locomotion, the cells reverted to their stellate form.

SEM studies with the mammalian NC too have revealed progressive elongation of the cells prior to migration(MORRISS & THOROGOOD, 1978). These flask-shaped cells often have microspikes on their surfaces and are connected to the apical end of the neural epithelium by elongated filaments.

TEM studies to elucidate the ultrastructure of the neural crest have unfortunately yielded very little information by comparison to

SEM studies. This is mainly due to the fact that migrating NC cells are often indistinguishable from surrounding mesoderm unless they are fixed as they are leaving their sites of origin. Moreover, there is little change in the NC cellular cytoplasm to set them apart during the early stages and cytoplasmic differentiation is believed to be a by-product of NC arrival at their migratory end-points(BANCROFT & BELLAIRS, 1976).

NEURAL CREST MIGRATION

ONSET OF MIGRATION

The appearance of the NC and its subsequent migration is spatially and temporally ordered in an anterior to posterior wave, at least in the avian(WESTON & BUTLER, 1966; BANCROFT & BELLAIRS, 1976; TOSNEY, 1978), and amphibian(LOFBERG & AHLFORS, 1977) embryo. That is to say, the neural crest cells of the anterior regions are the first to move out while migration occurs progressively later from regions further and further posteriorly.

In the avian embryo, the neural tube closes in an anteroposterior sequence(TOSNEY, 1978) and the initiation of NC migration has been shown to occur at the time of, or soon after, neural tube closure(WESTON, 1963, JOHNSTON, 1966). A similar pattern has been observed for the trunk NC in mammalian embryos(DERBY, 1978) but an important distinction must be made for the mammalian cranial NC. In the mammalian embryo, neural tube closure does not occur in a cranio-caudal fashion(as in avian embryos) but starts in the cervical region(in the region of 4th to 7th somites) and then spreading both anteriorly and posteriorly(HAMILTON & MOSSMAN,1972).

This has prompted the suggestion that the migration of the cranial NC
in mammalian embryos is not a function of neural tube closure but of
the age of the neural epithelium(JOHNSTON, 1965; MORRISS & THOROGOOD,
1978). As a corollary, the migration of the cranial NC in mammals need
not necessarily occur in an antero-posterior sequence.

MIGRATION PATHWAYS

It has been known for quite some time now that the migration of NC
cells is not a random arrangement but that well-defined and precise
pathways exist within the developing embryo(Fig. 3). As early as
1946, HORSTADIUS & SELLMAN used Nile-blue sulphate and neutral red to
demonstrate alternating streams of NC cells directed toward the visceral
arches in urodeles. Prior to that, DETWILER(1937) had used a vital dye
(Nile blue sulphate) with some degree of success to trace the migration
of NC cells in the trunk region.

Two pathways of cell migration are generally described; one beneath
the skin ectoderm close to the somites, and the other between the neural
tube and adjacent somites(Fig. 4). It is easy to see , even on a trans-
verse plane, that the pathways are restricted by the topographical
limits imposed by the neural tube, somites and the surface ectoderm.
These two basic pathways for the trunk NC have also been confirmed by
various studies using a variety of sophisticated methods including
^{3}H-thymidine labelling and inter-specific chimaeras.

The amphibian embryo in particular has been studied with great
success and CHIBON(1967) used ^{3}H-thymidine followed by autoradiography
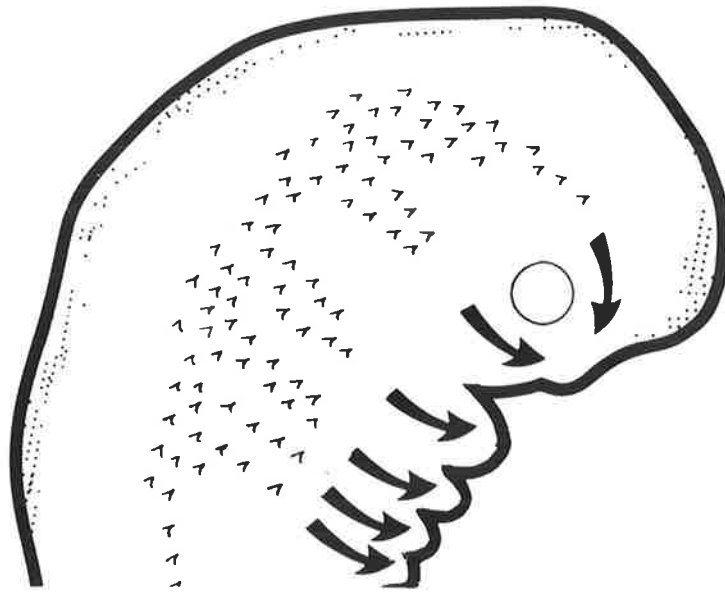
Fig.3 The migration of NC cells occur via well-defined
      physical channels in the ventrad direction indicated
      by the large arrows.
      (From Sperber, 1976).
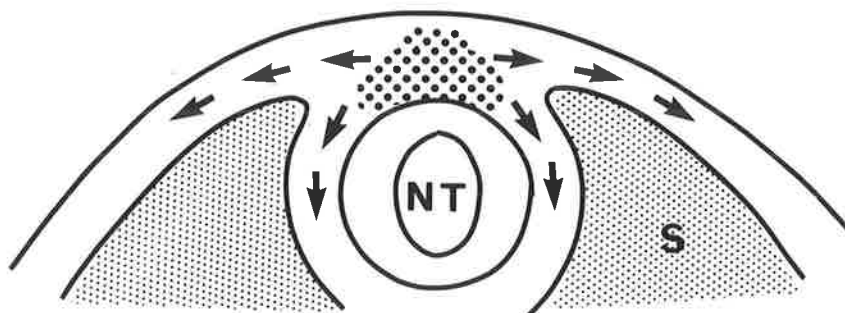
Fig.4 Two pathways of NC cell(large dots) migration in the
trunk region : dorsolateral and ventral.
Neural tube(NT), Somites(S).
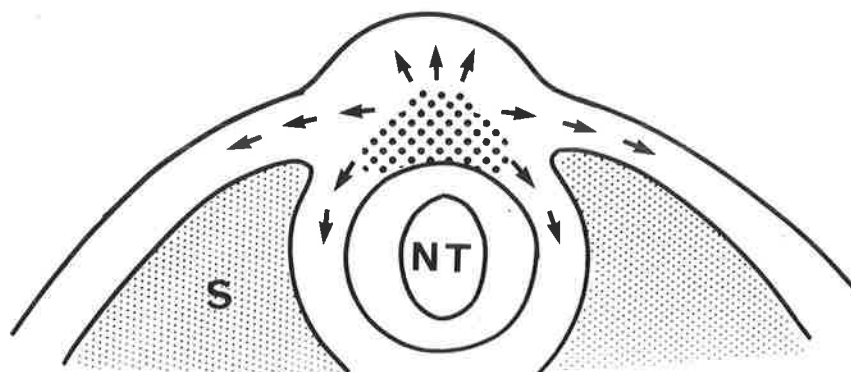
Fig.5 In amphibia, the trunk NC makes an additional contribution
to the dorsal fin mesenchyme, giving rise to a third
pathway in the dorsad direction.
Neural tube(NT); Somites(S).

to confirm and also to amplify the observations of HORSTADIUS & SELLMAN
(1946). It should be noted that the amphibian trunk NC is unique in its
phylogeny because it gives rise in addition to the usual derivatives,
an additional contribution to the dorsal fin mesenchyme. Hence, a third
migratory pathway has also been elaborated(Fig. 5)(LOFBERG & AHLFORS,1978).

In the avian embryo, ^{3}H-thymidine labelling followed by orthotopic
grafting has similarly been carried to map out the migratory fates of
both trunk(WESTON, 1963; WESTON & BUTLER, 1966) and cranial(JOHNSTON,1965;
NODEN, 1975) neural crest. In the trunk region, the migratory routes
were found to be in agreement with the basic pattern set out in Figure 4.
However, some interesting modifications described by WESTON(1963) merit
discussion. After leaving its initial position, the neural crest is
described as being split up into two streams, one following the dorso-
lateral route and the other the ventral route.

The dorso-lateral group of cells, generally acknowledged to be the
precursors of melanocytes(RIS, 1941; RAWLES, 1947) were found to penetrate
the surface ectoderm almost immediately and in this location they continued
their migration in an unsegmented fashion. This observation by WESTON(1963)
would corroborate the conclusion of TWITTY & NIU(1948), that the ectoderm
is the principal site for the migration of melanoblasts. Others however,
contend that NC cells migrate beneath the ectoderm and enter the differen-
tiating epidermis from the underlying mesoderm prior to pigmentation(RIS,
1941). This latter view has been confirmed by TEILLET & LE DOUARIN(1970)
who in chick-quail chimaeras, demonstrated the subectodermal pathway
of prospective melanocytes.

The ventral stream was described by WESTON(1963) as a population

migrating close to the neural tube in a uniform distribution until it reached the level of the somites whereupon migration was confined to the somitic boundaries and absent in the intersomitic areas. Consequently,a segmental pattern of neural crest cell migration was produced due to the metameric arrangement of the somites. In this study, the first ever to employ autoradiography, WESTON(1963) described the penetration of somitic mesenchyme by NC cells, a point confirmed by JOHNSTON(1965) and CHIBON(1967) who both worked on amphibian embryos and by BANCROFT & BELLAIRS(1976) in avian embryos. It is now generally accepted that this population of cells would eventually differentiate into spinal sensory ganglia and also the neurons of the autonomic nervous system.

Studies on the cranial NC have yielded almost identical migratory routes but because of the mesenchymal contribution by NC cells here, important differences do exist. JOHNSTON's work(1965, 1966) on the cranial NC in the avian embryo revealed that ^{3}H-labelled cells were finally localised in the mesenchymal regions of the head but apart from that, provided little information regarding the migration pathways. Similarly, due to dilution of the label by cellular proliferation, it was impossible to follow the fates of the NC cells up to the stage of differentiation. Thus, in spite of numerous claims(JOHNSTON, 1966; JOHNSTON & LISTGARTEN, 1972), ^{3}H-thymidine has never been demonstrated within the fully differentiated NC cells, e.g. chondrocytes although the label might be found surrounded by cartilage tissue. Fortunately, the problem is now resolved with the discovery of a permanent cell marker in the quail-chick chimaeric system(see NEURAL CREST CELL EXPERIMENTATION). Nonetheless, JOHNSTON(1965, 1966) has described two pathways in the craniofacial regions of the avian embryo; a dorso-lateral and a ventral pathway. The dorso-lateral pathway was said to be consisted by a group of NC cells migrating in the form of a sheet

beneath the surface ectoderm to terminate in the mesenchymal areas of the facial processes and the eye. The ventral pathway, contrary to other descriptions, was said to be located <u>within</u> the neural tube where they eventually become cranial nerve nuclei.

Although JOHNSTON's work with ^{3}H-thymidine has yielded valuable information regarding the terminal locations of migrating cranial NC cells, more benefit has been obtained from the use of autoradiography to map the spatial distribution of migrating NC cells. NODEN(1975) carried out a series of remarkable experiments with the chick embryo using a variety of orthotopic and heterotopic grafts to map out the migratory end-fates of the cranial NC(Fig. 6). By orthotopic grafting, a piece of neural fold after labelling with ^{3}H-thymidine is grafted into the equivalent anatomical site of the host whereas a heterotopic graft would be implanted into a non-identical site.

Orthotopic grafting was performed to trace the normal migration pathways and destinations of NC cells derived from each region of the neural tube(Fig. 6). Thus cells from the midbrain region(Areas 2 & 3) only took the dorso-lateral pathway beneath the surface ectoderm to contribute to the maxillary process mesenchyme. In contrast, NC cells from the hindbrain(Areas 4 & 5) took both pathways: dorsolateral to arrive at the mandibular and hyoid arches; ventral to arrive at the future sites of the cranial sensory ganglia.

Heterotopic grafting on the other hand yielded a different set of information altogether, namely to clarify if NC cells of each area are predetermined to follow a particular migratory route. The results showed that this was not the case and that physical channels defined

Possible Substrata	Destination
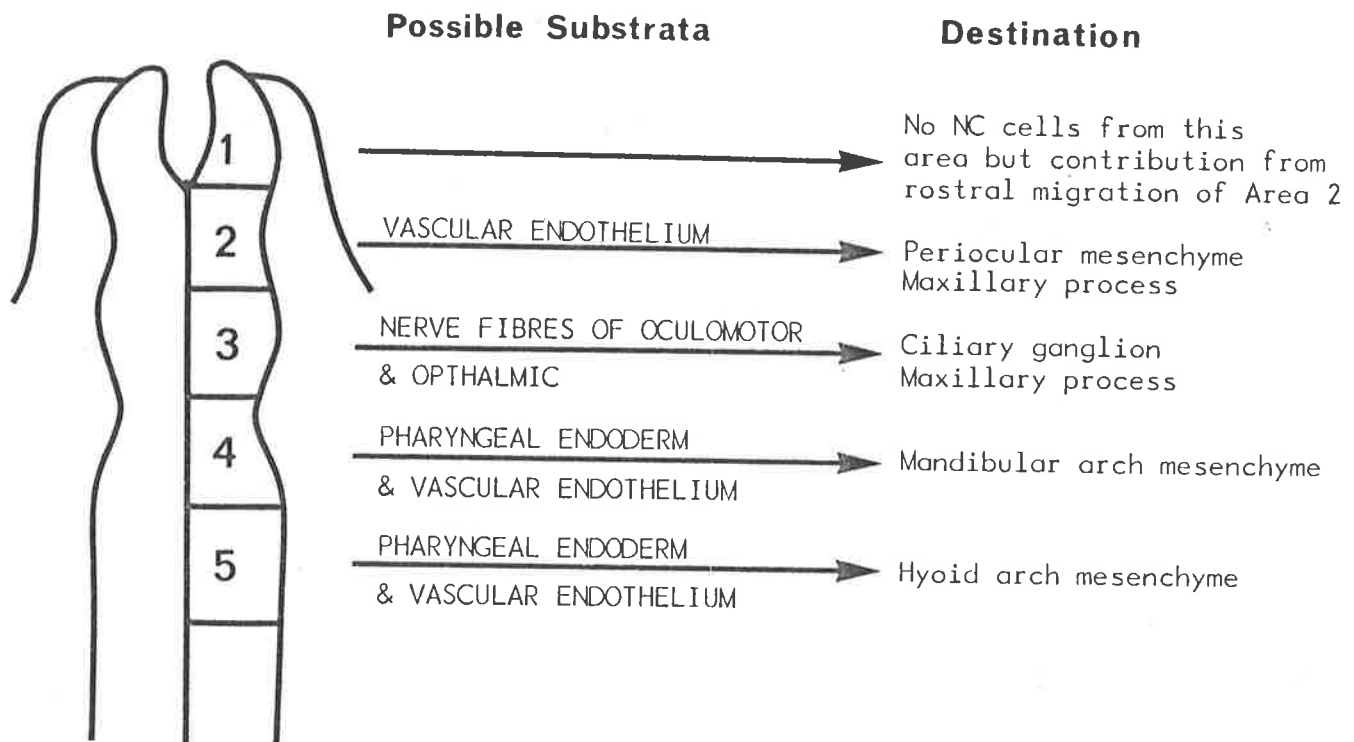
Fig.6 Migratory destinations of the cranial neural crest in
chick embryo(After Noden, 1975).
The cells arrive after travelling in the dorso-lateral
pathway(subectodermal), possibly making use of certain
anatomic structures as substrata.
The major regions of the brain are designated according to:
Area 1 : Prosencephalon
Area 2 : Diencephalon & anterior mesencephalon
Area 3 : Posterior mesencephalon
Area 4 : Metencephalon
Area 5 : Anterior myelencephalon

by the environment were responsible for the migration pathways observed.
For example, a labelled graft from area 2 when implanted into area 4
was found unable to migrate into the periocular regions(where it is
normally found) but instead behaved like area 4 cells, condensing into
the sites of cranial sensory ganglia and contributing to mandibular arch
mesenchyme. Thus, it is almost certain that irregardless of their site
of origin, NC cells have a capacity to recognize and respond to highly
specific environmental cues which direct not only their migration but
also their subsequent differentiation(see NEURAL CREST DIFFERENTIATION).

Having described the spatial configuration of migration pathways,
it now remains to mention the temporal factors affecting NC cell migration.
WESTON & BUTLER(1966) thought that migration pathways have a temporary
existence only, related to the onset of migration. To prove their point,
they showed that when 'old' NC cells were transplanted into 'young'
hosts undergoing NC migration, the full range of trunk NC derivatives
was produced. In contrast, 'young' NC cells failed to contribute to
either the sympathetic or spinal ganglia of the 'old' host when the
time difference was maximal(12 hours). These experiments would suggest
that some temporal alteration has occurred in the host environment and
this has limited the migration of NC cells subsequently implanted. This
view is contested by NODEN(1975) who felt that the failure of 'young'
NC cells to differentiate in 'old' hosts is due to the saturation
of 'distal niches' by early migrating host NC cells.

While the ability of a particular environment to support NC cell
migration may be temporally limited, studies have shown that the age
of the NC is not so critically defined. Apart from the work of WESTON &
BUTLER(1966) where 'old' NC cells were shown capable of migrating in

a 'young' environment, LE DOUARIN(1980) has very recently transplanted
ciliary ganglion(a fully differentiated NC derivative) into a sympathetic
area of the neural axis. Subsequent mapping of the quail graft showed
that the environment was capable of altering the developmental bias
of the fully differentiated ciliary ganglion from parasympathetic to
sympathetic. On the other end of the scale, NODEN(1975) grafted precocious
trunk NC cells into the cranial region and found that the cranial environment
was equipped to support normal migration.

POSSIBLE MECHANISMS OF DIRECTIONALITY

It has been observed that the pathways of NC cell migration are
highly specific and largely under the control of directional cues provided
by the environment. While the actual mechanisms of NC motility are poorly
understood, much has been suggested regarding the possible mechanisms
by which the cells are guided along a particular pathway.

NC cells at the start of migration always tend to move away from
their source(neural tube) and this phenomenon has consistently been
reported by all workers(HORSTADIUS, 1950; WESTON, 1970). Several
hypothetical explanations for this phenomenon have been suggested.

    a) mutual repulsion hypothesis

    b) changes in adhesive properties

    c) chemotaxis

    d) contact inhibition

    e) contact guidance

a) Mutual repulsion hypothesis

When amphibian NC cells are placed under coverslip glass in a fluid
medium, they tend to move rapidly away from one another. Single cells
when isolated in the same fluid medium exhibited amoeboid changes but
failed to migrate(TWITTY & NIU, 1954). It was therefore postulated that
a diffusible substance produced by the crest cells is responsible for
the repulsive force. This concept was supported by the independent
observations of HOLTFRETER(1968).

b) Changes in adhesive properties

This hypothesis has been forwarded by WESTON(1970) to account for
the migration and subsequent aggregation or dispersion of NC cells.
Adhesive interactions can occur between like-cells of the NC population
(homotypic) or between NC cells and cells of the environment(heterotypic).
Thus the net migration of NC cells away from the neural tube can be
explained as movement from a region of low adhesive interaction into
a region of more stable adhesive interactions. By similar reasoning,
changes in the mutual affinity of NC cells for each other would cause
a fall in homotypic adhesive stability leading to NC cell dispersion
as opposed to aggregation. Evidence for this theory has been provided
by the observation that cells of various tissues often have selective
preferences of adhesion when placed together in suspension(ROTH & WESTON,
1967; ROTH, 1968).

c) Chemotaxis

Since migrating NC cells have often been reported to be in close

association with pharyngeal endoderm prior to differentiation into cartilage, it is conceivable that a chemotactic substance might be produced by the pharyngeal endoderm to attract the NC cells. This hypothesis has been tested both *in vivo* and *in vitro* and found to be wanting. HORSTADIUS(1950) removed the left hind-brain NC and after staining it with Nile blue, grafted it on to the right ventral aspect of the head(close to the pharyngeal arches). The undisturbed NC on the right side was stained with neutral red to distinguish it from the graft. As migration proceeded, blue coloured cells from the graft were seen travelling in a dorsal direction away from the pharyngeal arches while red coloured cells were directed towards the pharyngeal arches, forming an alternating pattern of colours. It would appear that movement away from the neural tube was the sole preoccupation of the cells, irrespective of the site of grafting.

EPPERLEIN(1974) studied the question by *in vitro* experimentation and found that even when NC tissue was cultured together with pharyngeal endoderm on glass surfaces, the migration of the cells was random.

d) Contact inhibition

Contact inhibition, defined as the directional restriction of displacement on contact was originally proposed by ABERCROMBIE & HEAYSMAN (1954) to explain why fibroblasts in culture have a tendency to migrate out radially, forming a monolayer with the least number of intercellular contacts. This phenomenon has been used to explain NC cell migration *in vitro* (EPPERLEIN, 1974). If extrapolations can be made to the *in vivo* situation, it would account for the directionality of NC cells migrating from a region of high population density to a low population density

area(WESTON, 1970; EBENDAL, 1977; TOSNEY, 1978). However, as WESTON(1970)
pointed out, for contact inhibition to operate under *in vivo* conditions,
it will be necessary to assume that this type of cellular behaviour is
more pronounced between NC cells and less between cells of the NC
population and the surrounding environment.

Arguments for and against contact inhibition have also been raised.
BANCROFT & BELLAIRS(1976) observed that NC cells in migration often
overlap each other, in contrast with the typical monolayering of
fibroblasts in culture. However, it has been argued that this multilayering
of NC cells does not necessarily preclude contact inhibition movement
(TOSNEY, 1978; LOFBERG & AHLFORS, 1978). It has been shown that multilayering
can occur with migrating fibroblasts both *in vitro* and *in vivo* by the
temporary abolition of contact inhibition by extracellular material
intervening in between cells. In addition, the orientation and elongated
morphology of NC cells(TOSNEY, 1978; LOFBERG & AHLFORS, 1978) and the
frequent presence of electron-dense plaques between NC cells(EBENDAL,1977;
LOFBERG & AHLFORS, 1978) are observations consistent with the behaviour
of cells governed by the rules of contact inhibition movement.

e) Contact guidance

Contact guidance was first described by WEISS(1934) to account for
the orientation of cell locomotion as a response to the shape of the
substratum. Much of our understanding concerning the role of contact
guidance on migrating cells have been derived from studies on fibroblasts
in vitro. ELSDALE & BARD(1972) showed that when hydrated collagen gels
are drained to produce a lattice of oriented fibres, cells placed on the
fibrillar matrices produced a longitudinal orientation in the direction

of the fibres. DUNN & HEATH(1976) reported that when fibroblasts are cultured on glass fibres less than 100 um in diameter, the cells tended to elongate and orientate theselves parallel to the fibre axis. However, the same cell when placed on fibres of larger diameter tended to spread out and assume a multipolar shape.

The immediate vicinity of the NC during the early stages of migration is made up by surface ectoderm, neural tube, somitic mesenchyme and the intercellular spaces. If contact guidance was to play a major role in regulating the orientation of NC cell migration, then the substrata responsible for providing the guiding stimulus will have to come from some or all of these structures.

One of the first structures mentioned was the surface of the neural tube. WESTON(1963) thought that the tangential direction of NC departure was in some way due to contact guidance provided by the dorsal neural tube. Later, as the ventral stream arrived into the somitic area, the neural tube is again said to be responsible for guiding the entry of some NC cells into somitic mesenchyme. LOFBERG & AHLFORS(1978) re-examined this question in amphibia with a view to applying the hypothesis of DUNN & HEATH(1976) regarding the curvature of the substrata. It was not surprising to find that the curvature of the neural tube supported flattened NC cells compared with elongated cells elsewhere. The basal lamina of the neural tube has also been reported to establish contacts with the psuedopodial processes of NC cells(EBENDAL, 1977).

The somitic mesenchyme has been cited as an enhancer of NC cell migration responsible for producing a segmented effect on the ventral population and consequently producing a segmented arrangement of spinal

sensory ganglia. This segmental pattern was abolished when the neural tube was implanted into an environment devoid of metameric somites (WESTON, 1963).

BANCROFT & BELLAIRS(1976) have pinpointed the basal lamina as the most likely substrate responsible for contact guidance in NC cell migration. The neural tube, the surface ectoderm and the somites are known to be surrounded by a basal lamina although discontinuities exist around the surface of the somites(TEILLET & LE DOUARIN, 1970). These discontinuities would then serve as convenient points for the entry of NC cells into somitic mesenchyme(BANCROFT & BELLAIRS, 1976). Significantly, the basal lamina has previously been dismissed as a substrate for NC cell migration in the head regions(JOHNSTON & LISTGARTEN, 1972).

Both SEM and TEM studies have in the past, revealed the presence of matrix fibrils possibly collagenous in nature and associated with glycoproteins in the extracellular environment before and during the onset of NC cell migration. When found on the neural tube surfaces, the matrix fibrils were often oriented in the direction of NC cell departure and indeed, NC filopodia were often seen extended along the axes of the fibrils(LOFBERG & AHLFORS, 1978). The frequent incidence of such a relationship was considered more than concidence; a finding unfortunately not shared by others(BANCROFT & BELLAIRS, 1976; EBENDAL, 1977; TOSNEY, 1978). However, even though the observation of LOFBERG & AHLFORS(1978) in amphibia might be an isolated instance possibly attributable to taxonomy, the frequent observation of a randomly arranged fibrillar meshwork on the surfaces of chick neural tube and surface ectoderm deserve more consideration(BANCROFT & BELLAIRS,1976; EBENDAL, 1977; TOSNEY, 1978; STEFFEK, MUJWID & JOHNSTON, 1979). These fibrils were often in close contacts with NC cells and interstitial bodies(TOSNEY, 1978).

Until further evidence becomes available, the role of the matrix fibrils as an adhesion enhancer(STEFFEK et. al, 1979) or as substrata for contact guidance cannot be dismissed.

The later stages of NC cell migration have not been examined in as great a detail. However, NODEN(1975) has listed some structures frequently encountered by labelled NC cells in the head regions of the chick embryo (Fig. 6). These include pharyngeal endoderm, vascular endothelial walls and nerve fibres. This is an interesting observation because histochemical studies of NC cell migration in mammals have often reported an early appearance of a capillary network associated with the migrating cells(DALCQ, 1953: MILAIRE, 1959; POURTOIS, 1961). To be sure, GAUNT(1959) has described how the future position of a tooth(a NC derivative) along the oral epithelium can be predicted by the early appearance of a rich vascular network underneath . The vascular system could conceivably serve as 'highways' for the transport of NC cells in migration.

THE ROLE OF THE EXTRACELLULAR MATRIX(ECM)

The embryonic spaces through which early NC cell migration occur are for the most part, filled with amorphous extracellular matrices(ECM). This ECM is made up by various types of collagen, glycoproteins and a variety of glycoaminoglycans(GAG). Previously known as mucopolysaccharide, GAG are made up of a sugar acid(hexuronic acid) and an amino sugar (hexosamine). They can exist in the free state(e.g. hyaluronic acid) or in the sulphated form(e.g. chondroitin sulphate) become attached to protein residues forming proteoglycans. In recent years, the role of GAG in regulating morphogenetic processes has been given increasing prominence and the neural crest has been a favourite target of study. Apart from this, GAG have also been implicated in the control of proliferation (COHN, CASSIMAN & BERNFIELD, 1976), maintenance of morphogenetic structures (BANERJEE, COHN & BERNFIELD, 1977), cellular differentiation(MARKWALD, FITZHARRIS & ADAMS SMITH, 1975) and embryonic induction(MEIER & HAY, 1974).

It has been reported that the onset of NC cell migration is preceded by the formation of a cell-free space in between surface ectoderm and underlying mesenchyme(PRATT, LARSEN & JOHNSTON, 1975). This newly created space is the result of massive production of hyaluronic acid(HA) by surrounding structures: neural tube(HAY & MEIER, 1974), surface ectoderm (PRATT, MORRISS & JOHNSTON, 1976) and the NC cells themselves(GREENBERG & PRATT, 1977; PINTAR, 1978). Hyaluronic acid is capable of expanding its volume a thousand times on hydration(LAURENT, 1970) and is aptly suited as an inflatable matrix. A similar increase in HA content has been noted in other morphogenetic events; for example, in the invasion of corneal stroma by NC cells(TOOLE & TRELSTAD, 1971), and in endocardial mesenchyme(MARKWALD & ADAMS SMITH, 1972).

While the concomitant increase of HA with NC cell migration is a universally acclaimed phenomenon(DERBY, 1978; PINTAR, 1978, WESTON et. al, 1978), the appearance of the cell-free space seems to be confined only to the avian neural crest(PRATT et.al, 1975; EBENDAL, 1977). Others (WESTON et.al, 1978; MORRISS & THOROGOOD, 1978) have searched in vain for a similar cell-free space in trunk and cranial NC in mammalian embryos.

The significance of HA among migrating cells is a subject of wide discussion. TOOLE(1972) originally postulated that HA in its hydrated form might serve as a suitable substrate for cellular migration and that the accumulation of HA would produce an expansion of previously closed spaces to facilitate cell migration. As a corollary, the fall in HA content, either by enzyme degradation or decreased production would lead to arrest of cellular migration and the resultant condensation would encourage more intimate cellular contacts leading to tissue differentiation(TOOLE, 1973). In other words, the function of HA is not only related to cell migration but also to the postponement of tissue differentiation.

Besides HA, other forms of GAG have been associated with the initial and later stages of NC cell migration. During the onset of migration, the wedge-shaped spaces bounded by the somites, neural tube and surface ectoderm are often occupied by huge quantities of both HA and sulphated GAG(PINTAR, 1978; DERBY, 1978). With progressive migration, not only the amounts but also the kinds of GAG were found to change as the NC cell population traversed through different parts of the embryonic environment. Significantly, PINTAR(1978) found that under *in vitro* conditions, neural crest, somites and ectoderm were all capable of synthesizing HA and sulphated GAG. Interestingly enough, NC cells produced a higher level of

HA while sulphated GAG synthesis predominate in somite and ectodermal cells. This would confer upon the environment(and NC cells) with the power to modify, as they wish, the surrounding GAG content and composition. The net synthetic activity would then determine whether cells continue on their migratory journeys as a dispersed population(at high levels of HA) or coalesce to form a cell type such as sensory ganglia(at low levels of GAG). WESTON et.al(1978) have suggested that if the GAG levels were to remain elevated, then alterations in its composition would result in sympathetic ganglia formation.

By further extrapolation, the observed differences in GAG distribution have been credited with the role of determining the net direction of NC cell migration(WESTON et.al, 1978). These workers have also suggested that by mediation of cell to cell interactions, characteristic differences in GAG composition could influence the choice of phenotypic expression by a basically pluripotential NC population. In support of this concept, alterations of culture conditions(medium and substrate) are known to induce melanogenesis in cultures of nerve tissue(NICHOLS, KAPLAN & WESTON, 1977). More recently LE DOUARIN(1980) has reported that the factors, responsible for favouring either acetylcholine or catecholamine synthesis by NC cells in culture, are likely to be found in the culture medium. In short, could the much discussed environmental cues responsible for NC differentiation be found in the ECM. Current evidence would indicate so and further experimentation would be expected to provide a more definite answer.

NEURAL CREST DIFFERENTIATION

DERIVATIVES

It should be mentioned at the outset that the neural crest is not the only ectodermal structure capable of migrating into underlying tissues and contributing to other structures. The neural tube, optic cup and cranial nerve placode have all proved capable of migrating into deeper embryonic structures. Thus some doubt must be cast at grafting operations where arbitrarily defined limits have been drawn by the author; engendering the possible inclusion of epidermal placodes, for example, into the grafts.

When the results of experiments performed on lower vertebrates are carefully extrapolated, the NC is seen to give rise to a spectacular range of derivatives(Table 1). Many of the conclusions, originally drawn from autoradiographic studies, have since been confirmed in chick-quail chimaeras (LE DOUARIN, 1975).

Table 1

SUMMARY OF NC DERIVATIVES

TRUNK NEURAL CREST

PIGMENT	MESENCHYME	ENDOCRINE	NEURAL	
			SENSORY	*AUTONOMIC*

1.Melanophores
2.Xanthophores
3.Iridophores
	Dorsal fin mesenchyme
	  (in amphibia)
			1.Spinal ganglia
			2.Vagal root ganglia
			  (some contribution)
				3.Sympathetic ganglia
				4.Adrenal medulla
				5.Parasympathetic ganglia

			6.Supportive cells (glial & sheath) 7.Meninges	

CRANIAL NEURAL CREST

Small contribution only	1.Odontoblasts
	2.Membrane bone
	3.Cartilage
	4.Connective tissue
	5.Smooth muscle
		1.Carotid body
		  (APUD cells)
		2.Ultimobranchial body
		  (calcitonin cells)
		3.ACTH secreting cells
		4.MSH secreting cells
			1.Cranial ganglia	2.Parasympathetic ganglia
				
				3.Supportive cells
				  (glial & sheath)

THE QUESTION OF INDUCTION

By induction, signals are transmitted from one tissue type to another for the purpose of eliciting developmental changes. Such events in embryology are termed tissue interactions and it generally believed to occur between tissues at close range(HOLTFRETER,1968). Hormones for instance, tend to exert their influence over a long-range target. To be precise, induction is a one-way affair, with no feed-back available to the inductor(tissue producing inducer). It is probable that a single stimulation is all that is required to evoke a developmental change; unlike hormonal stimulation which requires for the maintenance of an event, a continuous supply(HOLTFRETER,1968). To be sure, a positive response to induction should be unambiguous and recognizable; taking the form of cellular division, changes in cell shape or specific protein synthesis(HAY,1976).

A useful working hypothesis has been framed by SAXEN(1977) to distinguish between directive and permissive influences in tissue interactions. Directive influences refer to those interactions in which the target cells possess more than one developmental option, and in which the inductive effect leads to selection of one of them. Permissive interactions refer to those in which one tissue acts upon another already determined towards its final fate, but which still requires an exogenous stimulus for the expression of its phenotype.

PRIMARY AND SECONDARY INDUCTION

The first tissue interaction in the developing embryo is generally known as primary induction. At the late gastrula stage, the ectoderm is

induced by the notochord and adjacent mesoderm to form the neural plate. Thus the neural crest is the product of primary induction and this has led many to assume that primary induction has in some way endowed the NC cells with the necessary information for the differentiation of pigment cells and neurons(HOLTFRETER,1968).

Subsequent tissue interactions that occur in embryogenesis are termed secondary inductive events. The neural tube for example, after primary induction, is in turn able to induce cartilage formation from somitic mesenchyme. According to the tissue types, secondary induction can be classified as:

epithelial-epithelial e.g. lens placode-optic cup

epithelial-mesenchymal e.g. corneal epithelium-NC ectomesenchyme

mesenchymal-epithelial e.g. NC ectomesenchyme-enamel epithelium

Thus while some NC cells are preprogrammed by primary induction to differentiate into certain cell types(e.g. melanocytes), others would require further tissue interactions and the exchange of secondary inducers prior to overt differentiation. In support of this, HOLTFRETER (1968) has reported successful cartilage formation from NC cells in culture but only after the addition of pharyngeal endoderm.

CAUSAL MECHANISMS OF NEURAL CREST DIFFERENTIATION

While primary induction might have caused some restriction of
potential(RAVEN & KLOOS, 1945), the majority of NC derivation still
rely on further tissue interactions before finally expressing their
phenotypes. Some of these interactions are encountered along the
migratory route while others are met on arrival at their migratory
end-points. The interactions can occur between adjacent NC cells
(homotypic) or between NC cells and surrounding tissues(heterotypic).
From what is known, the term environmental factors can be justifiably
applied to cover the motley of tissue interactions known to accompany
NC ontogeny. The precise nature ot these factors is a subject of wide
speculation and as many theories have been forwarded as there are NC
derivatives.

a) HOMOTYPIC INTERACTIONS

This is typified by the *in vitro* cultures of spinal ganglia.
COWELL & WESTON(1970) described a correlation between cell aggregation/
neurogenesis and cell dispersion/melanogenesis.A quantitative factor
is believed to be at work and in the *in vivo* situation, the aggregation
of NC cells in somitic mesenchyme is believed to trigger off spinal
ganglia differentiation(WESTON, 1970).

b) HETEROTYPIC INTERACTIONS

There are at present a few NC cell types known to be dependent upon
epithelial-mesenchymal interactions before they are capable of overt
differentiation. The derivation of sympathetic ganglia is known to be

the result of a prior association of NC cells with both neural tube surface
and somitic mesenchyme(COHEN,1972; NORR,1973).

Cartilage differentiation in amphibia has been shown to be dependent
upon an interaction of NC cells with pharyngeal endoderm during migration
(HORSTADIUS & SELLMAN, 1946). This inductive event has been confirmed
by the *in vitro* studies of EPPERLEIN & LEHMANN(1975) using neural fold
tissue and pharyngeal endoderm. They found that in TRITURUS, cartilage
differentiation occurred only at the points of contact between NC
and endodermal cells cultured adjacent to each other.

A further example is found in the differentiation of membrane
bone in the chick embryo. The *in vitro* formation of membrane bone
was observed only when epithelial-mesenchymal interactions between
mandibular epithelium and NC ectomesenchyme had previously transpired
(TYLER & HALL, 1977).

At the cellular level, the inductive signal can be transmitted
via cell-to-cell or cell-to-matrix contacts although a clear-cut
distinction is often not possible at the light microscopic level.
Evidence for cell-to-cell contacts is provided by EPPERLEIN & LEHMANN
(1975). They noted that cartilage formation *in vitro* was conditional
upon a heterotypic interaction bwtween NC cells and pharyngeal
endoderm. An initial inductive stimulus is believed to be transmitted
and subsequent spread of this inductive message is thought to occur
by homotypic cellular contacts between adjacent prechondroblasts.
Support for a cell-to-cell contact theory can be found in the transfilter
studies with a non-NC derivative: kidney tubules failed to form in the
absence of cell-to-cell contacts between metanephric mesenchyme and
a non-specific inductor such as spinal cord(WARTIOVAARA, NORDLING,
LEHTONEN & SAXEN, 1974).

There is more evidence to support the contention that a cell-to-matrix interaction is operative, at least under *in vitro* conditions. A diffusible substance released into the matrix by endodermal cells is thought to mediate cartilage differentiation(HOLTFRETER, 1968) and a soluble serum factor is believed to favour either sympathetic or parasympathetic expression by NC cells in culture(LE DOUARIN, 1980). NEWSOME(1975, 1976) on the other hand, argued against the diffusibility of the agent and showed that direct contact between NC cells and the ECM(secreted by pigmented retinal epithelium) was prerequisite before cartilage differentiation in chick sclera.

NATURE OF THE INDUCTIVE STIMULI

The precise nature of the inductive influences that apparently elicit differentiation in nascent embryonic cells and determine specific cellular functions is a matter of conjecture. In the experiments of NEWSOME(1975, 1976), the extracellular matrix *per se* was shown capable of exerting its inductive effect, even in the absence of the living retinal epithelium; which in normal conditions has been shown to secrete a collagenous, PAS-positive substance. However, substitution with other types of collagen did not invoke chondrogenesis in NC cells or NC-derived periocular mesenchyme. Hence it would appear that the extracellular matrix secreted by the pigmented retinal epithelium is fairly specific although the nature of the interaction is thought to be a permissive one rather than directive(NEWSOME, 1976).

Transfilter studies with yet another NC derivative has shed further light on the nature of the inductive stimuli. Tooth development is a result of a series of interactive events, commencing from the time of NC

migration. An association with pharyngeal endoderm is known to occur

in amphibia(HORSTADIUS & SELLMAN,1946) and at the time of their arrival

into the jaws, the NC ectomesenchyme are thought to be determined to

become odontoblasts. However, overt differentiation into odontoblasts

does not occur until the bell stage of tooth development and experimental

studies have shown that the differentiation of ectomesenchymal odontoblasts

and epithelial ameloblasts is the result of a series of tissue interactions

occurring in a definite sequence(KOCH, 1967, 1972; SLAVKIN, 1974).

Previously it has been observed that differentiation of NC ectomesenchyme

into odontoblasts did not occur in the absence of epithelium(KOCH, 1967).

This led to the hypothesis that a cell-to-cell interaction was responsible

for mediating the inductive stimuli. Since the basal lamina remains intact

throughout the duration of odontoblast differentiation(KALLENBACH,1971;

MEYER, STAUBLI, FABRE & RUCH, 1977), a cell-to-cell mediated inductive

stimulus would not be possible(THESLEFF, 1977b). Instead transfilter

studies have revealed that odontoblast differentiation is triggered off

by close contacts between NC ectomesenchyme and the extracellular matrix

associated with the basal lamina(THESLEFF, 1977a, b). The probability of

a secretory product equivalent to that in retinal epithelium(NEWSOME,1976)

was eliminated by transfilter studies(THESLEFF, 1978b). Since the basal

lamina is known to be composed of Type IV collagen and proteoglycans,

attempts were made to induce odontoblast differentiation with a variety

of collagenous substances; all without success(KOCH, 1975; THESLEFF, 1978b).

This has led to the conclusion that it is in the three-dimensional

structure of the basement membrane where the inductive stimulus is

likely to be found(THESLEFF, 1978b).

PLURIPOTENTIALITY

Barring a few possible exceptions - chondrogenic differentiation, cholinergic differentiation - it is almost certain that NC cells at the beginning of their migration are not committed to differentiate into any particular cell type. Instead, the final location and differentiative fate of a given NC cell population along the neural axis is the net result of environmental signals received en route.

The above is not to imply that all NC cells have the same degree of pluripotentiality prior to migration. Due to primary induction, some restriction of potential is inevitable(RAVEN & KLOOS, 1945) and it has been noted, for example, that while cranial NC is able to differentiate into cartilage, trunk NC is not(HORSTADIUS, 1950). Conversely, cranial NC has only a limited ability to form pigment(FOX, 1949). However, the delineation into trunk and cranial NC is more of a seamless boundary than a clear-cut definition. NODEN(1975) has shown that the more superior parts of the trunk NC are capable of behaving like cranial NC in the appropriate environment. More precisely, LE LIEVRE & LE DOUARIN(1975) have shown that up to the 5th somite level; trunk NC when correctly manipulated, is capable of giving rise to the full range of cranial NC derivatives.

It has been emphasized that the phenotypic expression of NC cells is a labile phenomenon. That is to say, the differentiative fate of NC cells in general is not a function of the cells' initial position along the longitudinal axis of the neural tube but dependent upon environmental cues encountered along a specific pathway. A series of experiments performed with the quail-chick system has served to illustrate the point. The trunk NC normally gives rise to ganglia of the autonomic nervous system and it

has been established that the parasympathetic ganglia of the gut is

normally derived from the 'vagal' NC(somites 1-7) and the sympathetic

ganglia from the NC corresponding to somites 8-28. More specifically,

the sympathoblasts of the adrenomedullary glands are derived from NC

between somites 18-24(LE DOUARIN & TEILLET, 1973). After heterotypic

grafting of vagal NC into the adrenomedullary area, sympathoblast

differentiation in adrenomedullary tissue was detectable. Similarly,

adrenomedullary NC when grafted into the vagal area produced normal

enteric ganglia with cholinergic differentiation. In sum, it was the site

of grafting and not the origin of the graft that determined that

fate of the cells.

Further experimentation was then performed to resolve one question:

are the environmental cues to be found along the migratory route, or

are they localised at the migratory end-points. The results would indicate

that for the trunk NC at least, both systems are operative; one for para-

sympathetic and the other for sympathetic differentiation. In direct

combinations of trunk NC tissue into the gut wall, thus circumventing the

migratory route, cholinergic activity was always the end result. This

would mean inductive influences along the migratory route, if any, can

be safely ignored with no loss of parasympathetic expression in NC cells

(SMITH, COCHARD & LE DOUARIN, 1977). However, it does not mean that

the inductive influences favouring parasympathetic differentiation are

only to be found at the gut tissues. In fact, mesencephalic NC cells

(destined for the ciliary ganglion, a parasympathetic NC derivative)

are shown to be possessed of cholinergic activity even before the onset

of migration(SMITH, FAUQUET, ZILLER & LE DOUARIN, 1979). Hence, it will

be more reasonable to conclude that the role of the environment in the

case of parasympathetic differentiation would be confined to stabilizing

and enhancing cholinergic activity(ZILLER, SMITH, FAUQUET & LE DOUARIN, 1979). If these cells were subject to a sympathetic pathway, then cholinergic activity would cease and instead adrenergic activity would ensue. Astonishingly, even NC cells that were complete in their migration process and were fully differentiated(as in the parasympathetic ciliary ganglion) were not immune when exposed to the environmental cues of a sympathetic pathway, producing adrenergic cells(LE DOUARIN, TEILLET, ZILLER & SMITH, 1978).

In the case of sympathetic differentiation, it is quite definite that environmental cues resident along the migratory pathways play an indispensable role. The chemical nature of the cues is uncertain but the sources of the signals have been traced to the ventral surfaces of the neural tube, the notochord and the somitic mesenchyme(COHEN, 1972; NORR, 1973; TEILLET, COCHARD & LE DOUARIN, 1978).

The grafting experiments of LE DOUARIN & TEILLET(1974) produced one puzzling finding. When the cranial NC of the quail was grafted onto the adrenomedullary area of the chick trunk; brain tissue, connective tissue and cartilage of quail origin were recovered. Given the limits of pluripotentiality, it is still significant that chondrogenesis was able to proceed in spite of these NC cells having never encountered pharyngeal endoderm. Perhaps in higher vertebrates, an interaction with endoderm is not a requisite event in chondrogenesis(EPPERLEIN & LEHMANN, 1975; worked with amphibians). In confirmation of this, neural tubes have been shown to chondrify when cultured directly *in vitro* or grafted into chick chorio-allantoic membrane(HALL, 1979).

This last point has led some to favour a concept of NC heterogeneity within a given NC population rather than pluripotentiality existing within every single NC cell(MORRISS & THOROGOOD, 1978). By this rationale, the

NC is said to be pluripotential not because every cell has the capacity of giving rise(in the appropriate environment) to the full range of derivatives but instead within any level of the neural axis, the NC population is made up by cohorts of different genotypes: some mesenchymal, some neurogenic/melanogenic and others endocrine. Depending on the limits imposed by the environment, some cells would differentiate while others become aborted. The difference between the two definitions of NC pluri-potentiality is more than a quibble and unfortunately refractory to existing methods of experimental analysis where whole populations, and not single cells, are studied.

SUMMARY

It is fairly certain that NC cells are not committed to follow a selected migratory pathway but their eventual location within a particular pathway is a function of the cells' initial position along the longitudinal axis of the neural tube. Physical channels of migration actually exist at different levels of the embryo and depending upon the nature of the environmental cues received en route, the differentiated end-product of the pluripotential cells is related to the pathway it has travelled. Finally, evidence would indicate that while a particular migratory route is only temporally related to the onset of migration, the ability of NC cells to change their developmental bias is a labile phenomenon.

NEURAL CREST EXPERIMENTATION

In the 1930s and 1940s, a great deal of experimental work was carried out in amphibian embryos. These experiments often involved techniques that had inherent shortcomings and the results were often ambiguous and contradictory. Lately, the avian embryo has been utilized with greater success in combination with sophisticated cell labelling procedures such as autoradiography and inter-species grafting. The mammalian is relatively unexplored except for histochemical studies. There are basically four methods of NC experimentation:

a) ablation

b) explantation

c) cell-labelling

d) histochemical staining.

a) ABLATION

The simplest procedure was to excise a part of the neural fold (containing the future NC cells) and observe what parts of the embryo failed to develop(HARRISON, 1904; STONE, 1922). As pointed out by WESTON(1970), this technique makes two false assumptions:

1) that the ablated tissue contain the NC responsible for a particular derivative

2) the remaining neural crest tissue and surrounding structures(lateral ectoderm) are incapable of restoring the ablated portion by regulation. These,coupled with the fact that the NC cells could have migrated before extirpation make the results appear rather equivocal. In addition, this method does not account for migration pathways and hence environmental signals that are vital for the differentiation of some phenotypes.

b) EXPLANTATION

By explantation, a portion of the neural crest is extirpated and its developmental fate studied under certain defined culture conditions; such as the chick coelom, chorio-allantoic membrane(CAM) or just grown as organ cultures *in vitro*. This approach is capable of unravelling some of the NC phenotypes but the results can also be misleading. The main reason is again the environmental signals that are to be encountered along the migratory routes only. Hence, the failure of some NC cell-types to differentiate could be attributed to the absent inductive signals. In addition, the composition of the culture media and substrata is vitally important: horse serum favours acetylcholine synthesis while foetal calf serum enhances catecholamine synthesis(LE DOUARIN,1980). *In vitro* conditions are also known to promote melanoblast differentiation by dispersing the NC cells which under *in vivo* conditions, would aggregate to become ganglioblasts(COWELL & WESTON, 1970).

c) CELL-LABELLING

Cell-labelling in combination with *in vivo* experimentation has so far produced the most fruitful results, at least in lower vertebrates. There are essentially four varieties of cell markers:
1) natural cell markers
2) vital dyes
3) radioactive markers
4) inter-species chimaeras

     (1) and (4) employs features intrinsic to the NC.

     (2) and (3) involves the application of cell markers which are

               non-deleterious and easily visualized.

1) <u>Natural cell markers</u>

In the earlier studies, the NC was identified solely by histological criteria. In amphibia, NC cells possess pigment and little yolk particles (which soon disappear); setting them apart from the mesoderm whose cells contain large yolk globules but no pigment(LANDACRE, 1921; STONE, 1922; DE BEER, 1947). Others(RAVEN, 1937; TRIPLETT, 1958) made use of nuclear size and morphology to distinguish NC cells. Such an approach is only useful in regions of NC aggregation; but where NC dispersion occurs, as it often does, the individual cellular characteristics become difficult to identify.

2) <u>Vital dyes</u>

Intra-vital dyes;such as Nile blue sulphate, neutral red, may be applied *in situ* to a specific site of the embryo or the stained piece of tissue may be grafted onto an unstained host embryo. The migration of the cells followed by the dye can be identified by direct visualization (through the skin ectoderm) and fate-maps of NC migration constructed (HORSTADIUS, 1950). The main disadvantage is their lack of specificity and the dye can easily be transferred to neighbouring cells causing spurious labelling. Quite often, the dye disappears before the cells reach their destinations making identification impossible.

3) <u>Radioactive markers</u>

In avian and mammalian embryos, the NC cells remain indistinguishable as they migrate through the surrounding tissues. Radioisotopes are useful when introduced as an artefact into the NC cells, allowing some

measure of identification. Tritiated thymidine, a nuclear marker, has been used with a high degree of success with the minimum amount of deleterious side-effects(WESTON, 1963; WESTON & BUTLER, 1966, JOHNSTON, 1965, 1966; CHIBON, 1967; NODEN, 1975). Regulation by surrounding NC following ablation does not occur since the empty space is occupied by an equivalent structure from the labelled donor. Tissue interactions are also allowed to proceed as the labelled cells migrate through the host. However, this method suffers from the serious disadvantage of being diluted as extensive cellular multiplication of the NC cells occur. In other words, the label is not a permanent one and by the time of NC differentiation, the cells would be unidentifiable by autoradiography.

4) Inter-species chimaeras

The problems associated with the preceding techniques are all nearly circumvented with inter-species chimaeras. This approach relies on specific differences in cellular morphology and staining characteristics to distinguish host cells from donor cells. It is a permanent cell marker because the distinguishing characteristics are self-multiplying. This technique, first tried by RAVEN(1937), has undergone considerable refinement and the work of LE DOUARIN(1973, 1980) has in the last decade made significant additions to our understanding of NC migration and differentiation. In the quail/chick system, the quail nucleolus is distinguish by its large heterochromatin content at interphase - a situation without equal in other embryos. The unusual amount of heterochromatic DNA renders the quail nucleolus particularly visible when stained by the Feulgen-Rossenbeck technique(light microscopy), or uranyl acetate-lead citrate if electron microscopy is desired.

d) HISTOCHEMICAL STAINING

Considering the difficulty inherent in research with mammalian NC, this approach was the most popular and in many cases, the only one available. Histochemical demonstration of NC cells is based on the assumption that during migration and subsequent differentiation, chemical substances associated with these events are produced. With increasing acceptance of the concept of induction, many attempts were made to decode the chemical organizers that were responsible for morphogenesis. Ribonucleic acids were quickly singled out(BRACHET, 1957; DALCQ, 1960) because of their accepted roles in cellular duplication. By this interpretation, areas rich in RNA were identified with active morphogenetic events in the developing embryo.

Another substance was alkaline phosphatase, an enzyme closely allied with protein synthesis during embryogenesis. MOOG(1943) found that the cells of chick neural tubes were abundantly possessed of alkaline phosphatase. This information was quickly applied to mammalian embryos where alkaline phosphatase staining was used to describe NC migration in the branchial arches(MacALPINE, 1955).

In more extensive studies of craniofacial development in rodents, glycogen and mucopolysaccharides were added to the list(DALCQ, 1953; MILAIRE, 1959; POURTOIS, 1961). In 17 day rat embryos, tracts of mesenchyme, highly vascularized and rich in RNA and mucopolysaccharide were reported in the jaw regions(DALCQ, 1953). MILAIRE(1959) claimed that NC cells of the mouse embryo are rich in RNA and glycogen when compared to the surrounding mesenchyme. Using this approach, he traced the migration of NC cells into the maxillary and mandibular processes

in 8 to 10 day old mouse embryos(Figures 7 & 8). In one of the mandibular
arch, tracts of condensed NC cells subsequently differentiated into
Meckel's cartilage and others were found in close relationship with the
dental lamina. POURTOIS(1961) extended the observations and found that
large concentrations of glycogen, RNA and alkaline phosphatase were
often present in the nascent regions of tooth development.

More recently, NOZUE(1974a,b,c) claimed to have unravelled the
histochemical characteristics of neural crest cells in mouse embryos.
By a combination of staining methods(modified Bodian's stain, PAS reaction)
and tests for enzyme activity(alkaline phosphatase, acetylcholine esterase),
he ventured to describe the histochemical characteristics of NC cells.
In the same study, NC cells were described as being vulnerable to drugs
including Mitomycin C, hydrocortisone acetate and nitrogen mustard.
These being the criteria, the distribution of NC cells in mouse embryos
15 days old was described(NOZUE, 1974c). Unfortunately, the illustrations
are not particularly convincing and one will have to conclude that the
claims are premature.

In the last decade, acetylcholinesterase(AChE) have been discovered
to be present in abundant quantities through out the embryo, quite apart
from the nervous system. Elevated levels of AChE were often found in
areas known to be actively morphogenetic and the enzyme was sometimes
found in areas of active cell movement. This led to the conclusion that
AChE is involved somehow in the process of differentiation itself(DREWS,
1975). This finding was consistently reported throughout a wide range
of embryonic tissues from vertebrates(amphibian, avian and mammalian
embryos). As for the NC, AChE activity was found only in the migrating
NC cells of chick embryos(DREWS, 1975).
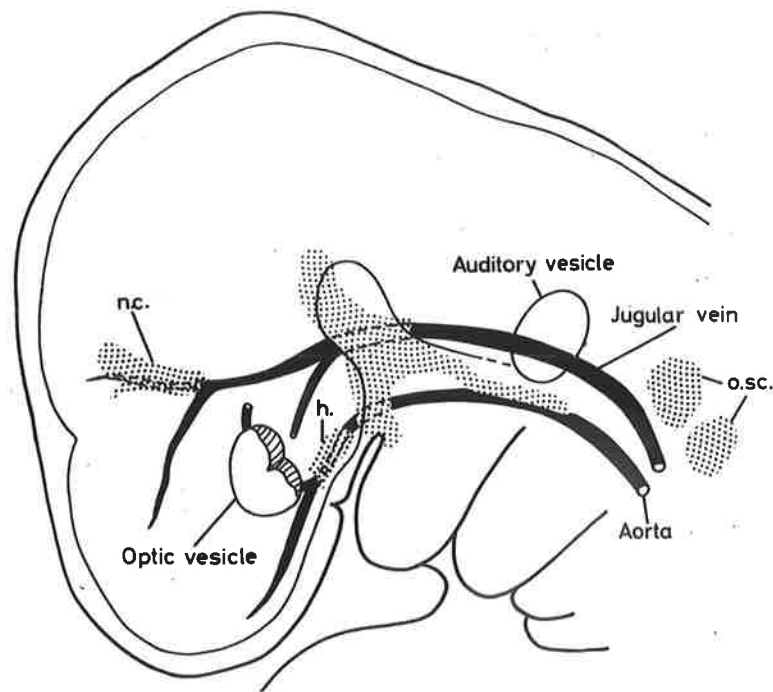
Fig.7 Diagram of lateral view of the head region of 10-day
       mouse embryo showing the relation of glycogen-rich
       mesenchyme(stippled areas) to the principal blood
       vessels. n.c., neurocranium h, hypophysis, o.sc.,
       occipital sclerotomes. (From MILAIRE, 1959).
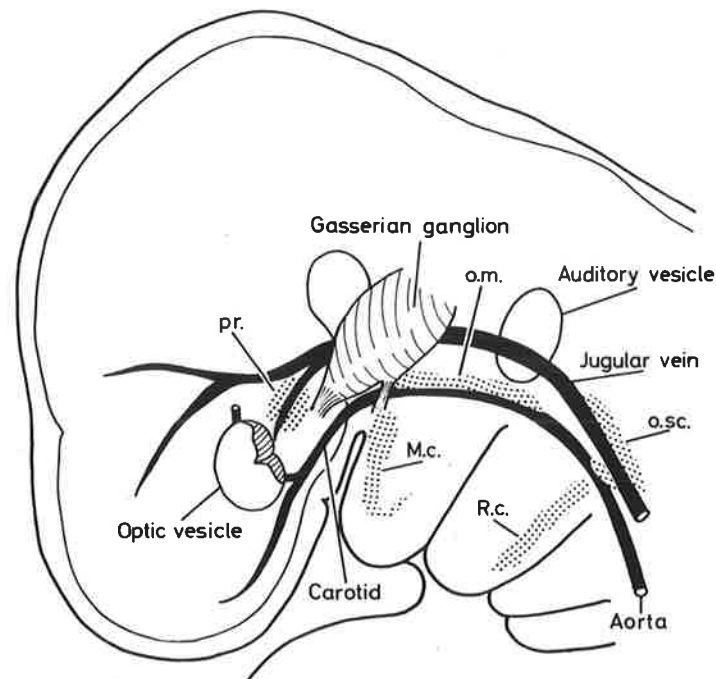
Fig.8 Diagram of lateral view of the head region of 10-day
       mouse embryo showing(stippled) areas of mesenchyme
       rich in alkaline phosphatase. p.r.,premandibular area,
       o.m.,occular muscles; M.c.,Meckel's cartilage;
       R.c., Reichert's cartilage. (From MILAIRE, 1959).

At first, many workers(e.g. JOHNSTON, 1965) were quick to dismiss histochemical methods as being unreliable. In the light of present evidence, some of the above studies may prove to be essentially correct. As an exemplar, the increased concentrations of extracellular GAG (mucopolysaccharide) in areas of active NC cell migration has been confirmed with more sophisticated methods in avian(PRATT et.al, 1975) and mammalian (DERBY, 1978) embryos. Nevertheless, demonstration of NC cell migration and differentiation by histochemical means alone is not without pitfalls. Substances such as alkaline phosphatase, glycogen, RNA and AChE are known to be widely distributed through out the embryo and their levels are often elevated in areas of active morphogenesis besides NC dispersion.

NEURAL CREST EXPERIMENTATION IN MAMMALIAN EMBRYOS

Up to the present moment, our information concerning the migration
and subsequent differentiation of mammalian NC has been derived
'secondhand' from non-mammalian vertebrates. Apart from a few descriptive
studies(HALLEY, 1955; BARTELMEZ, 1960), little experimental work has been
undertaken with mammalian NC. This is because viviparity has isolated the
mammalian foetus into an inaccessible position, making direct manipulation
of the NC difficult if not impossible. The other problem has to do with
the search for a permanent cell marker equivalent to the quail-chick
chimaera.

Apart from the aforementioned histochemical studies, some attempt
has been made to characterise mammalian NC with limited success only.
JOHNSTON(1965) used intra-vital dyes in rabbit and found that the basic
pattern of NC migration was similar to amphibian and avian embryos.
Since the study was considered preliminary, no photographs(apart from
a schematic diagram) accompanied the claim. A similar approach was adopted
in rat embryos(MORRISS & STEELE, unpublished) but was unsuccessful due to
the spread of the dye into areas adjacent to the point of application.
In an unpublished study(JOHNSTON & KRAMES), better success was reported
with rat embryos developing in whole embryo cultures through a 24 hour
period. ^{3}H-thymidine labelling followed by autoradiography revealed
silver grains in the mandibular arch area, a position known to be occupied
by chick NC cells(JOHNSTON & LISTGARTEN, 1975; JOHNSTON & PRATT, 1975).

Recent attempts to study alterations in GAG composition and
distribution among NC cells have been more fruitful with the mouse
embryo(DERBY, 1978; WESTON et. al, 1978). Sophisticated methods including

histochemical staining at critical electrolyte concentration(CEC), enzyme degradation and ^{3}H-glucosamine incorporation followed by autoradiography revealed that changes in morphogenetic behaviour of NC cells are accompanied by changes in GAG complexion.

There is also a similar paucity of experimental work on NC derivation in mammals and the purported NC origin of craniofacial mesenchyme remain an unverified hypothesis. Several studies on non-mesenchymal derivatives of the NC were more successful, most notable are the pigment studies of RAWLES(1947) and MAYER(1973,1975,1977). Using the approach of RAVEN (1937), RAWLES(1947) grafted pieces of tissue including skin ectoderm plus the underlying mesoderm, somites with and without the adjacent neural tube into the coelom of white leghorn chick embryos. The explants were taken taken from mouse embryos of a pigmented(black) strain, in this way, a piece of tissue can be removed before and after the predicted NC 'invasion'. When the grafts were subsequently recovered, only grafts containing NC cells or neural folds produced pigment. A piece of ectoderm taken before NC arrival would only produce white coloured hair but if removed at a later age, would produce black hair. These studies not only removed all doubts concerning the putative origin of melanocytes but by the use of grafts obtained from differently aged embryos, the rate of NC cell migration could even be mapped out(RAWLES, 1947). Such a discovery paved the way for a genetic analysis of pigment differentiation using a wide range of mutants with abnormalities in coat colour(MAYER, 1975, 1977). Hence, defects of pigmentation in 'piebald' mice were found to be located within the NC itself while in 'steel' mice, the NC cells were normal but abnormalities in the dermal environment precluded their differentiation. This approach, if properly applied, holds considerable promise for the analysis of other NC-derived cell types in mammalian embryos.

The use of chemical teratogens is another method of NC experimentation in mammalian embryos. The administration of excess Vitamin A into pregnant dams at day 8 to day 10 of pregnancy has been known to induce craniofacial abnormalities in rat embryos(MORRISS, 1972; 1973; POSWILLO, 1975). This stage of pregnancy is concurrent with the differentiation of the neural ectoderm and closure of the neural tube. Hence, any interference with normal development is attributable to abnormal NC migration and differentiation. The precise mechanism of Vitamin A teratogenecity is unknown, but there is a sufficient body of evidence to indicate that it interferes with the cellular mechanisms of locomotion. These findings, coupled with the observation that the period of greatest susceptibility (to Vitamin A teratogenecity) often coincide with NC migration, have led to the speculation that the rate and onset of cell migration is delayed(MORRISS, 1975). POSWILLO(1975) on the other hand, favoured a selective destruction of NC tissue theory. Studies in teratogenesis have yielded valuable insights on the nature of Vitamin A-induced craniofacial anomalies, but the interpretation of these experiments must necessarily rely on evidence derived from non-mammalian NC.

In recent years, the elucidation of NC migration and differentiation in mammalian embryos has become a real and exciting possibility; thanks to the whole embryo culture techniques of NEW(1967; see review NEW, 1978). The *in vivo* development of the rat embryo relies for its nutrition, the yolk sac placenta, until the 17-somite stage when the chorio-allantoic placenta links the foetal vessels to the maternal circulation. Whole embryo culture systems are quite capable of supporting the yolk sac placenta(and hence normal foetal metabolism) but not quite sophisticated enough to mimic a maternal circulation normally provided for by the allantoic placenta. This does not detract from the potential usefulness

of whole embryo culture techniques. As a matter of fact, it will be quite sufficient for our purpose since NC development, migration and early organogenesis would be expected to fall with the 'successful' period. The rat embryo can be maintained under *in vitro* conditions, starting from the primitive streak stage up to the 16-somite stage for at least 32 hours and even up to 48 hours although less successfully. Technique wise, the mammalian embryo - hitherto inaccessible to manipulation without the risk of damage to the enclosing vesicles - can now be subject to NC labelling, surgical grafting and maintained in culture before autoradiography(MORRISS & THOROGOOD, 1978). The result of such a study is eagerly awaited to improve our understanding of NC ontogeny in mammalian embryos.

THE NEURAL CREST IN ODONTOGENESIS

EVIDENCE FROM NON-MAMMALIAN VERTEBRATES

As early as 1898, PLATT(working on Necturus) thought that dentine
and pulp were derived from NC cells. This idea was supported by both
ADAMS(1924) and DE BEER(1947) who made independent observations on
the salamander(Ambystoma). STONE(1926) made use of ablation experiments
to show that teeth on the side of NC extirpation were considerably reduced
in number. RAVEN(1935), by xenoplastic transplants found Ambystoma NC cells
in the dental papilla of Triturus. Further experiments conducted by
WAGNER(1949) in chimaeras(Triturus & Bombinator) led to the same conclusion.

HORSTADIUS & SELLMAN(1946) found a reduction in the number of teeth
when grafting experiments disturbed the NC-derived tooth-bearing cartilages.
This led to the conclusion that the NC ectomesenchyme of dental papilla
is resident within the same regions responsible for cartilage formation.
In a further study, SELLMAN(1946) went so far to to delineate the NC zones
responsible for tooth formation in amphibia(Zones 3 & 4, Fig. 9).

The question of induction was quickly brought up in the attempts
to explain tooth and cartilage differentiation. To test the concept of
secondary induction, SELLMAN(1946) grafted pieces of NC tissue, accompanied
by various combinations of oral ectoderm and pharyngeal endoderm, into the
trunk regions. It was soon found that cartilage production required the
presence of pharyngeal endoderm; but for teeth production, both pharyngeal
endoderm and oral ectoderm have to be present in the grafts. These results
suggest a secondary inductive event between NC cells and pharyngeal

endoderm prior to chondrogenesis and in the presence of oral epithelium,
odontogenesis as well. The nature of this interaction has never been
fully understood and an equivalent event is generally assumed to occur
in mammalian embryos too.

THE NEURAL CREST IN MAMMALIAN ODONTOGENESIS

What evidence have we to suggest that NC cells fulfill an odontogenic
role in mammalian embryos? Unfortunately, very little. While the putative
NC-origin of amphibian odontoblasts has been proved beyond reasonable
doubt, extrapolations based on homology(SLAVKIN, 1974; JOHNSTON, 1976;
OSBORN & TEN CATE, 1976) must necessarily be met with objections.

The amphibian teeth when present, are basically epidermal caps
consisting of dentine covered by a layer of calcified enameloid.
No true gomphosis occurs(as in mammals) but attachment is gained by
a rigid union with the underlying bone(anchylosis; NOBLE, 1969). In
other amphibia, teeth arises out of cartilaginous jaws, a situation
without equal in mammals(WOERDEMAN & RAVEN, 1946). The amphibian dentition
is polyphyodont, that is, it exhibits a continuous succession of teeth.
Replacement of dental units occurs by new teeth arising from a persistent
dental lamina. Finally, as DE BEER(1947) has pointed out, amphibian teeth
can arise from either oral ectoderm or endoderm(defying the germ layer
theory) whereas mammalian teeth have a confirmed ectodermal participation
only. Then again, the tooth germ in lower vertebrates projects up into
the ectoderm whereas in higher vertebrates, the ectodermal layer grows
down into the mesenchyme. These differences are mentioned to emphasize
that genuine discrepancies between mammalian and amphibian teeth do exist,
making extrapolations from one to another unreliable.
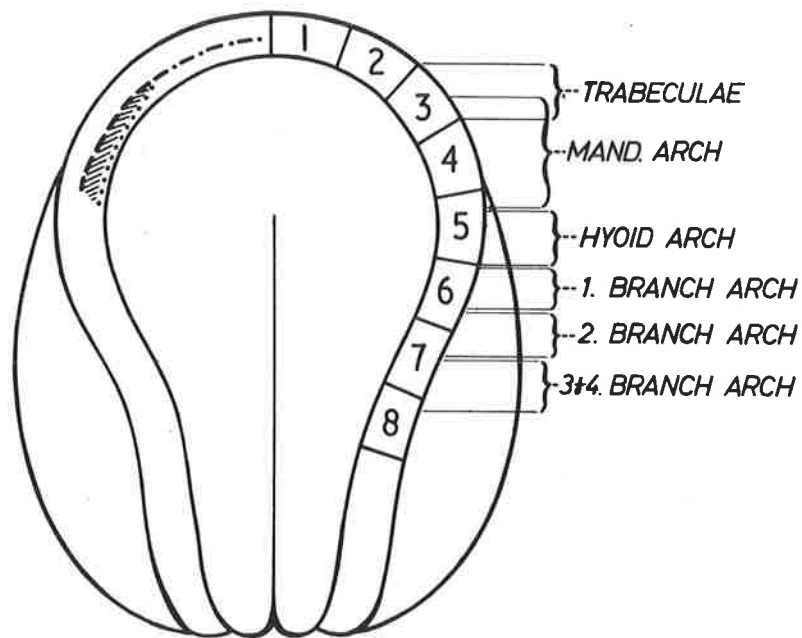
Fig.9 Schematic representation of the neural plate, surrounded
    by the neural folds, in the early neurula. The position
    of the presumptive areas of the head region which furnish
    ectomesenchyme to the respective visceral arches are
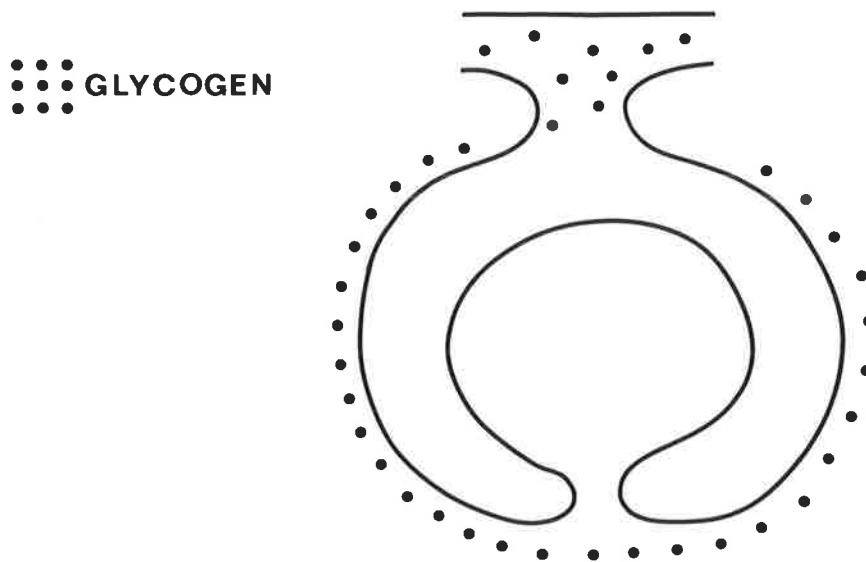   indicated on the right. (From SELLMAN, 1946).

Fig.10 The tooth germ at bell stage is surrounded by glycogen
    rich cells at the sites corresponding to the future
    dental follicle(After POURTOIS, 1961).

The avian embryo is arguably closer to the mammalian embryo on the evolutionary ladder, but unfortunately, is phylogenetically dispossessed of teeth. Hence, any conclusion drawn from avian studies, no matter how sophisticated the techniques of experimentation, has little relevance with regard to mammalian tooth formation. One must refrain from coming to any inferences concerning mammalian tooth formation by observations of ^{3}H-thymidine label in NC cells found within the cephalic mesenchyme of avian embryos. Such conclusions(JOHNSTON & LISTGARTEN, 1972; JOHNSTON, 1976) would appear, even to the casual observer, to be a curious *non sequitur*.

TEN CATE(1969, 1972) has done much to elucidate the development of the mammalian periodontium. He is convinced that the periodontium; comprising of ligament, cementum and alveolar bone, is of NC origin. A histochemical approach to the problem was adopted following observations of glycogen in dental tissues by MILAIRE(1959) and POURTOIS(1961) and any glycogen-containing cells in the vicinity of the developing tooth germ was identified as being of NC origin(TEN CATE, 1969). It would come as a non-surprise to find a distribution of glycogen-rich cells corresponding to the future positions of the dental follicle. These same cells were later found to differentiate into fibroblasts in the periodontal ligament(FREEMAN & TENCATE, 1971). A similar distribution of glycogen in developing teeth has previously been described by POURTOIS(1961; Figure 10). Thus, based on the link of glycogen content alone, NC cells are said to give rise to periodontal ligament(TEN CATE, 1972), cementum (TEN CATE, MILLS & SOLOMON, 1971) and alveolar bone(TEN CATE & MILLS, 1972).

The work of MILAIRE(1959), POURTOIS(1961) and TEN CATE(1969) have raised a potentially illuminating line of enquiry regarding NC participation

in mammalian odontogenesis. If, as they claimed, the NC population is
distinguished by the glycogen content; then the demonstration of this
polysaccharide in the vicinity of the dental rudiments could be
constituted as circumstantial evidence of ectomesenchyme migration.
Previous studies utilizing this approach have been unsatisfactory for
two reasons. First, the material used is often a tooth germ in a fairly
advanced stage of differentiation and the actual migratory event is not
demonstrable. Second, the identification of NC cells by their glycogen
content is inadequate by histochemical means alone. Glycogen will have
to be present in large quantities in the appropriate polymers before
they can be positively stained(PEARSE, 1960). Ultrastructural identification
of glycogen in cells is far more sensitive and reliable. In addition, such
an approach will allow the description of features currently thought
to be associated with cell motility. This is the rationale adopted by
the present study.

Chapter 4. MATERIALS AND METHODS

C3H mice were used for the study. Adult mice, placed together overnight for mating, were in the morning examined for the presence of a vaginal plug(KALLMAN, 1967). The midnight was designated the beginning of day 0. The age of the embryos were assessed by the number of days in gestation, but more accurately by the staging criteria of GRUNEBERG(1943). The pregnant dams were killed by cervical dislocation and the gravid uteri rapidly removed by surgical dissection. The individual embryos were then freed from the decidua and immediately transferred onto a dissecting stage.

PART 1. LIGHT MICROSCOPY

The heads of embryos $12\frac{1}{2}$, $13\frac{1}{2}$ and $14\frac{1}{2}$ days post-conception were detached from the rest of the body with a scalpel blade. The rest of the embryo was discarded.

FIXATION

The embryonic heads were immersed into neutral buffered formaldehyde(10%) solution and fixed for at least 48 hours.

DECALCIFICATION

This was achieved with a solution made up by equal parts of 40% Formic acid and 7% Sodium Formate. The tissues were allowed to remain in this solution for a week at the most.

EMBEDDING

The standard procedure of dehydration, infiltration and embedding in paraffin wax was adopted(Appendix 1).

SECTIONING

The foetal heads were carefully orientated to the plane of sectioning so that serial sections, each 7 µm in thickness, were obtained in a coronal plane. A LEITZ rotary microtome was used for the purpose.

STAINING

Sections collected on glass slides were stained with the Periodic acid-Schiff reaction(McMANUS, 1946). The staining procedure is outlined in Appendix 2. After clearing in xylene, the stained sections were mounted in a synthetic resin medium(dibutylphthalate in Xylol).

PART 2. TRANSMISSION ELECTRON MICROSCOPY(TEM)

A selected area of the foetal jaws, representing the molar tooth bearing areas, was subjected to ultrastructural scrutiny. The preparation of the specimens, outlined below, is identical for tissues obtained from both 12½ and 13½ day old embryos.

TISSUE REMOVAL AND FIXATION

Specimens were removed from the posterior areas of the palatal shelves, at the sites of the nascent maxillary molars beneath the developing eyes. The jelly-like tissues were gently lifted off from the rest of the maxillary process and immediately immersed into the primary fixative kept in an ice-bath. This fixative was basically made up of a phosphate buffer(pH 7.4) containing 3% glutaraldehyde(TAAB Laboratories, Berkshire, U.K.) in solution. In a few specimens, the fixative was augmented with 2% Tannic acid(Ajax Chemicals, Sydney).

After primary fixation, lasting for about 2 hours, the specimens were rinsed in phosphate buffer and post-fixed in Osmium tetroxide(1%) (Matthey Garrett & Co.,Sydney) for an hour. The fixative was then pipetted off and the remaining traces of osmium washed off with double distilled water.

DEHYDRATION

Dehydration was carried out by subjecting the specimens to increasing strengths of graded alcohol(Appendix 3). After this, the specimens were placed in two changes of propylene oxide(Ajax Chemicals, Sydney), each change lasting for approximately 15 minutes.

EMBEDDING

After the second change of propylene oxide, some more fresh propylene oxide was added to completely submerge the specimens. To this, an equal volume of EPON 812(LADD Research Industries Inc., Burlington, Vermont) was added. The vessel was gently swirled to ensure an even mixture of the EPON(Mixture A:B = 6:4). At the end of one hour, the specimens were individually embedded in Teflon capsules filled with fresh EPON mixture. The plastic was left to set in an oven set to $60^{o}C$ over a 48 hour period.

MICROTOMY

The blocks were removed from the capsules and after orientation, excess plastic trimmed away with a razor blade. Using a SORVALL MT-2 ultramicrotome, sections ½ µm thickness were obtained and stained with toluidine blue (mixture of 1% borax and 0.05% toluidine blue in equal parts) until the desired epithelial-mesenchymal interface was attained.

Thin sections with silver to gold interference colours were cut with glass and diamond knives, collected on copper grids(200 mesh) previously cleaned with glacial acetic acid and absolute alcohol.

STAINING FOR ELECTRON MICROSCOPY

For increased contrast, a double staining procedure was adopted.

a) URANYL ACETATE

The copper grids were placed section side down, on a drop of 2% uranyl acetate solution kept in a dust-free petri dish and previously

filtered through a Millipore syringe. After 20 minutes(WATSON, 1958), the grids were thoroughly rinsed in freshly boiled distilled water.

b)LEAD CITRATE

The grids were similarly treated with lead citrate(pH 12) stain for 12 minutes. At the end of this time, the grids were washed with 0.1 N Sodium hydroxide solution and double distilled water. The grids were then dried on filter paper and examined under a Philips 300 electron microscope operated at 60 kV.

PART 3. ORGAN CULTURE

For organ culture experiments, an aseptic technique is essential and all procedures were carried out under a culture hood(Biohazard, Clemco). Mouse embryos, 12½ and 13½ days in gestation, were freed from the decidua and washed in Hank's balanced salt solution(Commonwealth Serum Laboratories, Melbourne) - Appendix 4. For tissue extirpation, each embryo was laid on a dissecting stage under a stereo-viewing microscope and the mandible, together with the rest of the body was discarded.

The topographical limits of the nascent maxillary molars were defined by arbitrary means(after information gleaned from light microscopy in PART 1) and extirpated according to the following procedure. First, a coronal incision(Figures 11&12) to divide the incisor from the molar regions of the lateral palatal shelves. Then a horizontal incision on each side of the head just beneath the level of the developing eye, continuous with the coronal incisions just inflicted(Figures 13 & 14). The resultant blocks of tissue encasing the presumptive molar areas were then lifted out and washed in Hank's balanced salt solution for a minute.

The culture system consisted of a Millipore filter(0.45 µm porosity; Millipore Corporation, Bedford, Massachusetts) supported on plastic organ culture dishes(60 x 15 mm in diameter, Falcon Plastics, Oxnard, USA) filled with a chemically-defined medium(Fig. 15). A schematic representation of the culture set-up is found in Figure 16.

The explants were placed on the surface of the Millipore strip,

Fig.11
Palatal view of the maxilla
showing the palatal(P) and
septal processes.
Mouse embryo, 13½ days
postconception.
(Fast-green stain, x30)

1mm

Fig.12
Schematic drawing of the
maxilla showing the coronal
incisions. The molar(M)
bearing areas are contained
within the incisions(arrows)
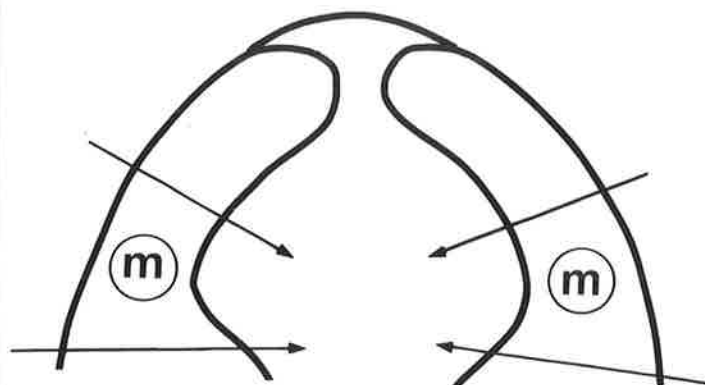
Fig.13
Lateral view of mouse embryo
13½ days post conception.
Tongue and mandible removed.
(Fast-green stain, x20)
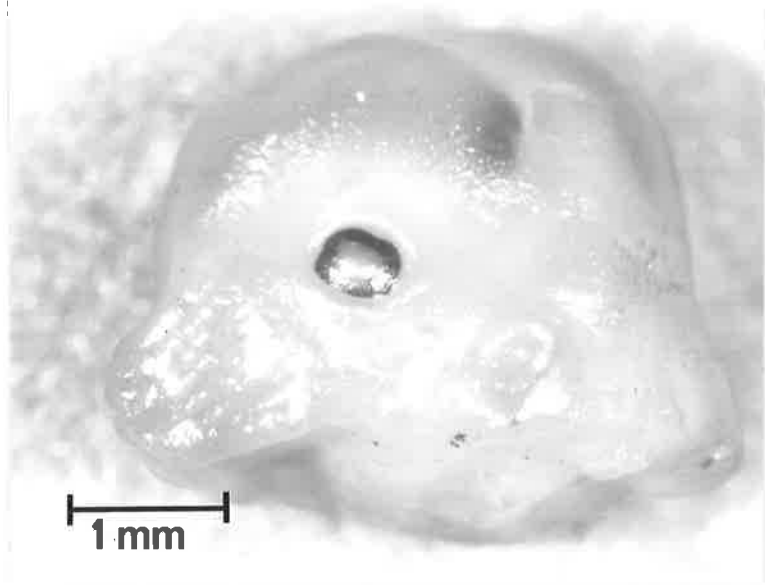
Fig.14
Schematic representation
of horizontal incision
(arrow) made beneath the
developing eye(E), join-
ing the coronal incisions.
The presumptive molar(M)
bearing area is contained
within the incisions.
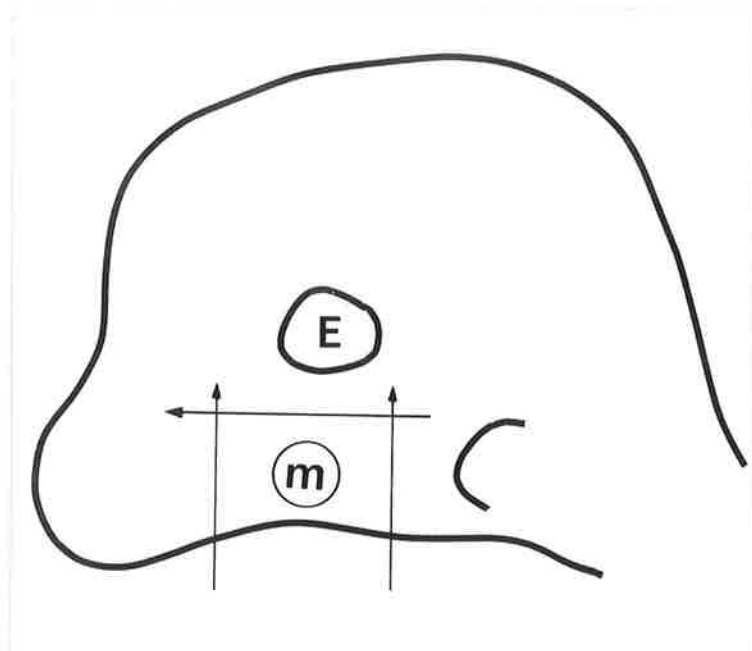
Fig.15 Explants(arrow) supported by Millipore filter saturated
by culture medium.
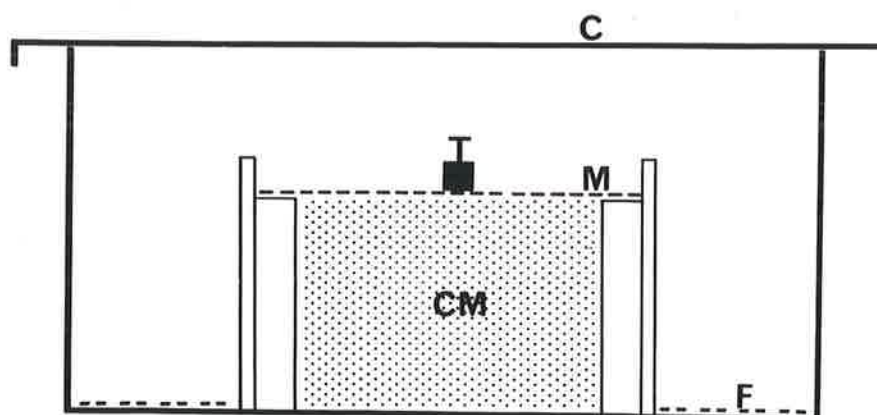
Fig.16 Schematic representation of culture set-up.
Tissue(T), Millipore(M), Culture Medium(CM), Filter paper(F)
Cover(C).

thus ensuring a gas-medium interface. Each Millipore strip carried two
pieces of tissue, occasionally three, but the explants were never placed
in close contact to one another. The identification labels were tagged on
to the lids and the cultures maintained at 37°C inside a humidified
incubator(Labmaster); gassed with a mixture of 95% oxygen and 5% carbon
dioxide. The medium was replaced every 48 hours and the gaseous atmosphere
replenished at each change.

Two separate studies were conducted, in each case, a different
medium compositon was employed. However, in both studies, the explants
were obtained from two dams: one pregnant for $12\frac{1}{2}$ days and the other for
$13\frac{1}{2}$ days. To ensure that the embryos in each litter were not developmentally
precocious or retarded, a single embryo from each litter was used as
a zero-time control and the nascent maxillary molar areas removed for
microscopic examination(TEM).

In the first study, the culture medium was Medium 199(Commonwealth
Serum Laboratories, Melbourne) - composition in Appendix 5 - supplemented
with 10% foetal calf serum(Commonwealth Serum Laboratories) and
with penicillin(100 U/ml) and streptomycin(100 µg/ml). The explants
were grown for a limited time period only, not exceeding 15 days at the
most. The cultures were harvested according to the plan set out in
Table 2.

In the second study, the main objective was to encourage hard tissue
formation *in vitro*. Previous reports of success achieved with tooth
germs cultivated at bell-stage(WIGGLESWORTH, 1968; THESLEFF, 1976)
prompted the choice of BGJ's medium(BIGGERS, GWATKIN & HEYNER, 1961).
For this purpose, BGJb medium(Fitton-Jackson modification; GIBCo, Grand

Island, New York) - Appendix 6 - was employed with supplements of foetal calf serum(20%) and Kanamycin(50 μg/ml). Apart from the change of culture medium, the rest of the experimental design remained basically unaltered from Study 1. However, the explants were grown for an extended period of time, and harvesting was done according to the plan set out in Table 3.

The cultivated tissues were fixed by immersion(together with the Millipore filter) in 10% normal buffered formaldehyde for 24 hours. Decalcification was achieved with a solution containing equal parts of Nitric acid(5%) and Sodium Formate(7%) for a period of 48 hours. The tissues were then processed in the usual manner, embedded in paraffin, and sectioned at 7 μm thickness. After staining with Haematoxylin and Eosin(Appendix 7), the sections were examined for the presence or absence of teeth.

Table 2 - Fates of explants in Study 1

	$12\frac{1}{2}$ day embryos	$13\frac{1}{2}$ day embryos
Zero time control	2	2
Harvested at 7 days	4	4
Harvested at 10 days	4	4
Harvested at 15 days	4	4
Total no. of explants	14	14

Table 3 - Fates of explants in Study 2

	$12\frac{1}{2}$ day embryos	$13\frac{1}{2}$ day embryos
Zero time control	2	2
Harvested at 15 days	12	2
Harvested at 16 days	-	2
Harvested at 17 days	-	2
Harvested at 18 days	-	2
Harvested at 19 days	-	2
Harvested at 20 days	-	1
Harvested at 30 days	-	1
Total no. of explants	14	14

Chapter 5. RESULTS OF THE INVESTIGATION

Part 1 - Estimation of dental development with the light microscope.

Part 2 - Ultrastructural examination under electron microscopy(TEM).

Part 3 - Results of organ culture.

PART 1. LIGHT MICROSCOPY

Serial sections of embryonic heads, cut in the coronal plane, were examined for PAS-positive material.

12½ day old embryos

The primitive oral cavity was bounded by the mandibular and maxillary processes(Fig. 17). The palatal shelves of the maxillary processes were widely separated, the septal process intervening in between. The mandibular process carried with it the tongue; the nerve and the artery of the future mandible were found lying within the mesodermal compartment of the first branchial arch. The developing Meckel's cartilage, and the septal process, were all darkly stained by PAS positive material.

A coronal section of the head at the level of the lens placode revealed a continuous layer of epithelial cells, lining the primitive oral cavity(Fig. 18). The earliest indication of tooth formation was evident by this stage, in the form of a localised proliferation of the oral epithelium - constituting the primary epithelial bands. This was true for both the maxillary and mandibular processes. The development of the epithelial band in the maxilla was found to lag behind that of the mandible. Thus, while the mandibular epithelial thickening was

observable at a particular region of the jaws(Fig. 19), no epithelial

band was seen in the maxilla. At other sites, both the maxillary and

the mandibular epithelial bands were present(Fig. 20). An indication of

the eventual bucco-lingual relationships was also given at this stage:

the maxillary epithelial band lying buccal to the mandibular band.

The oral epithelium was generally made up by a layer of low

columnar cells, and in the regions overlying the potential tooth bearing

areas, there was a more superficial layer formed by flattened cells.

At the site of the thickened epithelial band, the cells were mainly

cuboidal and up to 5 layers in thickness. The mesenchymal areas under-

neath were signally free of PAS-positive material, appearing to be

undifferentiated and of random arrangement(Fig. 21). However, the

thickening of the primary epithelial bands around the jaws was not

a uniform phenomenon, becoming totally indistinguishable in some areas

(Fig. 22).

Fig.17
Primitive oral cavity bounded
by maxillary and mandibular
processes. The palatal shelves
(P) are widely separated by
the tongue(T). Meckel's
cartilage(M), Eye(E), Neuro-
vascular bundle(NV).
Coronal section, 12½ days
post coitum. (PAS, x30)
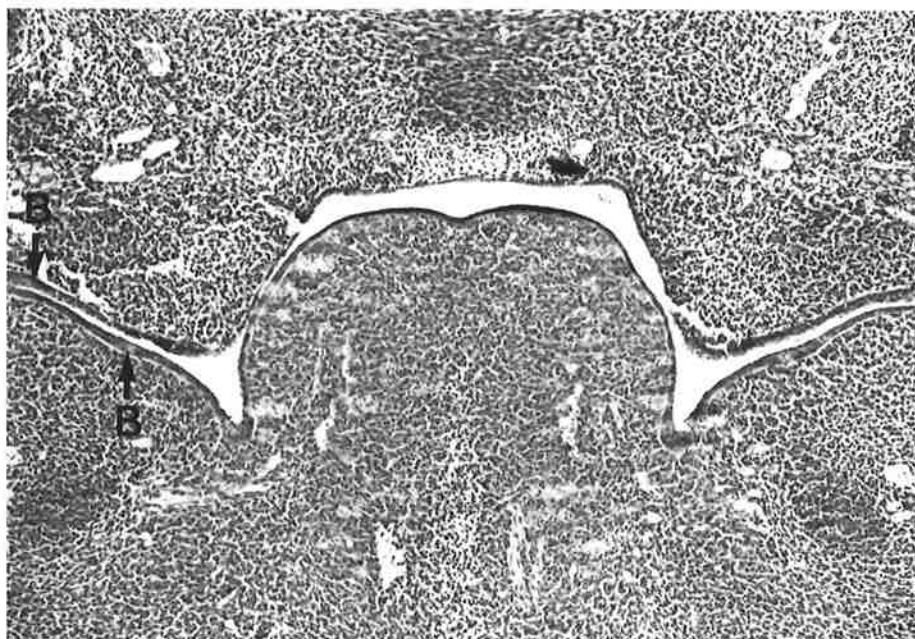
Fig.18 Coronal section at the level of the developing eye
    Primary epithelial bands(B) are seen in both arches.
    12½ day old embryo. (PAS, x60)
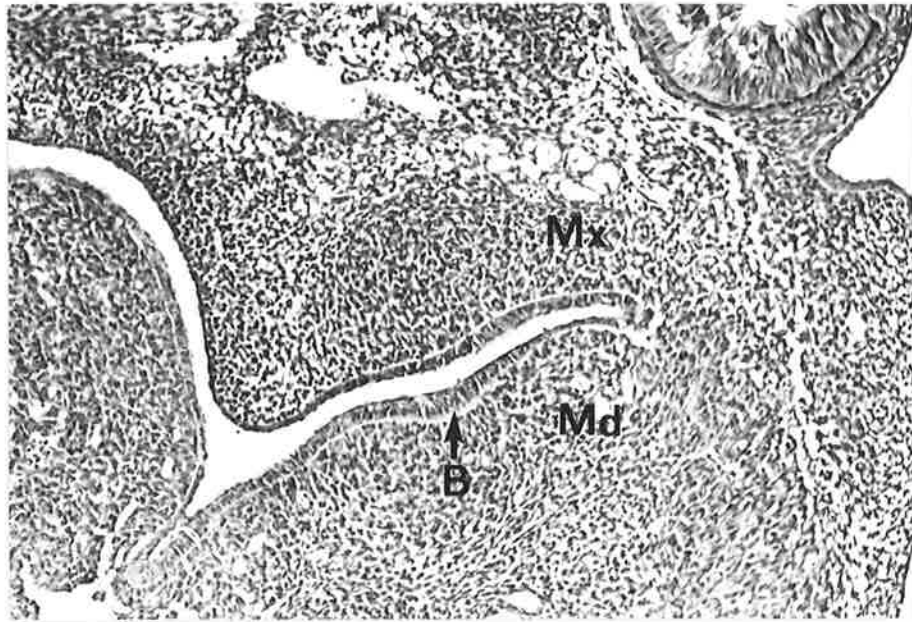
Fig.19 Primary epithelial band(B) present in the mandible(Md)
but absent in the maxilla(Mx).
Coronal section, 12½ day old embryo. (PAS, x100)
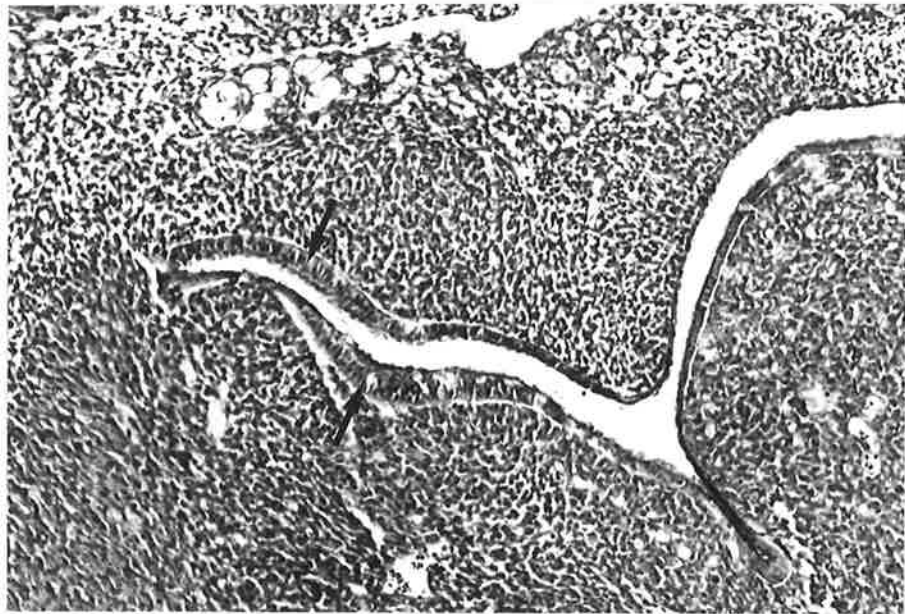
Fig.20 Thickening of the oral epithelium(arrows) seen in both
jaws. Coronal section, 12½ day old embryo.(PAS, x120).
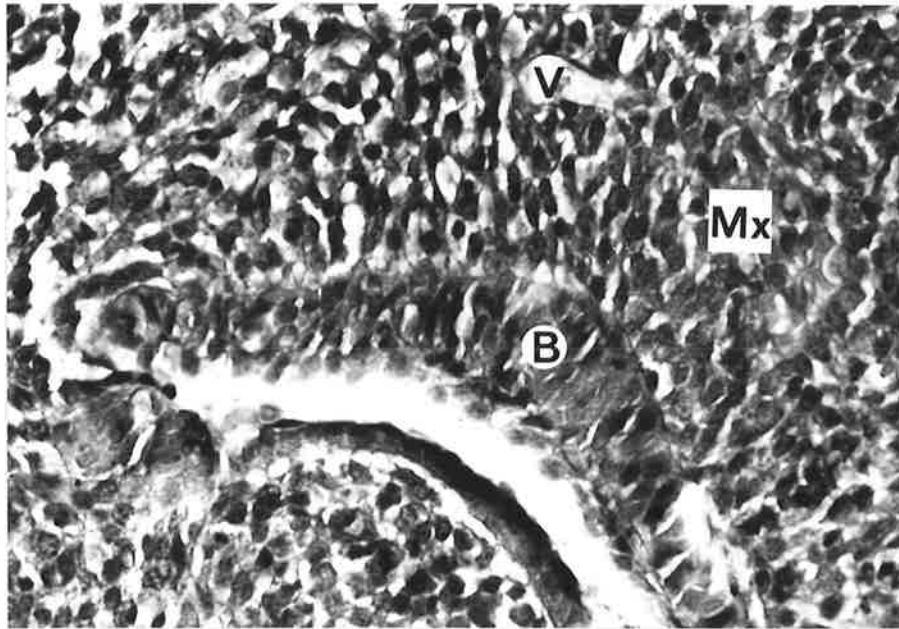
Fig.21 Primary epithelial band(B) of the maxillary(Mx) process.
At its thickest portion, the cells are 5 layers in thick-
ness. Surface layer of cells flattened. Note blood vessel
(V). Coronal section, 12½ day old embryo. (PAS, x400)
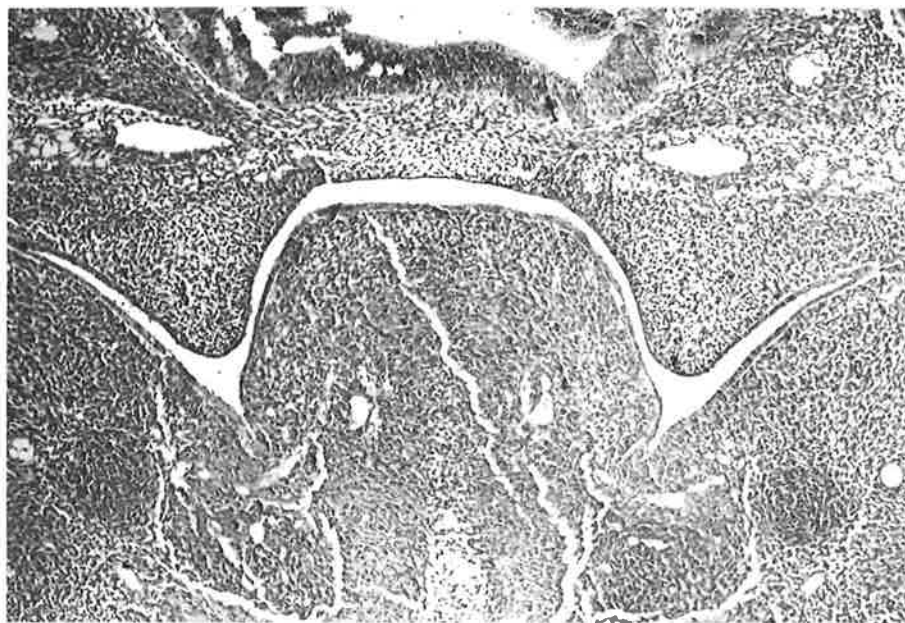
Fig.22 In some areas, the primary epithelial bands become
totally indistinguishable from the rest of the oral
epithelia. Coronal section, 12½ day old embryo.(PAS,
x75)

<u>13½ day old embryos</u>

The palatal processes were observed to be widely separated by the
intervening tongue(Fig. 23). The cells within the mesodermal layers
of the maxillary and mandibular processes revealed increased staining
density; in particular, the cells of the septal and Meckel's cartilages.
The cells of the oral epithelia were also darkly stained and vacuolated
areas were found within cytoplasm(Fig. 24). Definite areas of tooth bud
formation were evident in the mandible, connected to the dental lamina
by a short stalk(Fig. 25).

In the maxillary process, the epithelium had thickened perceptibly,
forming an ingrowth into the mesoderm(Fig. 26). It was difficult, at
this stage of development, to ascertain if the rounded ends were in
fact tooth germs or just part of the dental lamina. The basal layer,
made up by cells having a columnar appearance had nuclei that were more
or less situated at the apical ends of the cells. This gave rise to a
clear zone of lightly stained cytoplasm next to the underlying mesodermal
cells. More significantly, the mesodermal cells had undergone an
orientation in relation to the dental bud; appearing as a whorl-like
condensation around it(Fig. 26).

Numerous mitotic figures were observable within the cells of the
dental lamina and also in the adjacent mesenchyme. PAS-positive cells
were also seen within the mesodermal tissues, closely related to the
epithelium. The cells on the oral surface of the dental epithelium,
originally cuboidal in shape had by now taken on a squamoid appearance,
not unlike those lining the remaining parts of the oral cavity.

Fig.23
PAS-positive cells are seen
within the mesodermal tissues
of the facial processes. The
cells around the tooth buds
are also darkly stained
(arrows). Coronal section,
13½ day old embryo.
(PAS, x30).
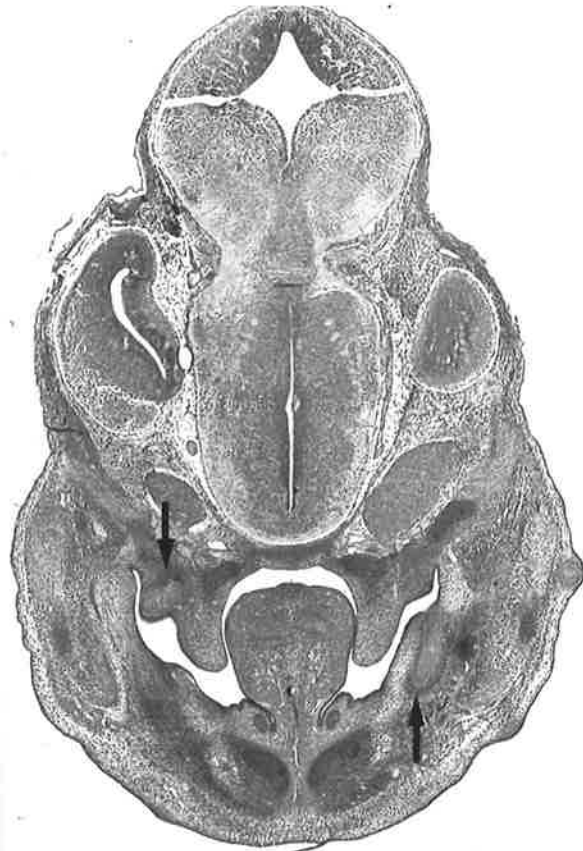
Fig.24 The cytoplasm of the epithelial cells(E) are darkly stained
due to their high glycogen content. Other cells appear vacuo-
lated due to the escape of the glycogen during tissue prepara-
tion. Note darkly staining cells of the mesoderm around the
tooth bud. (PAS, x200)
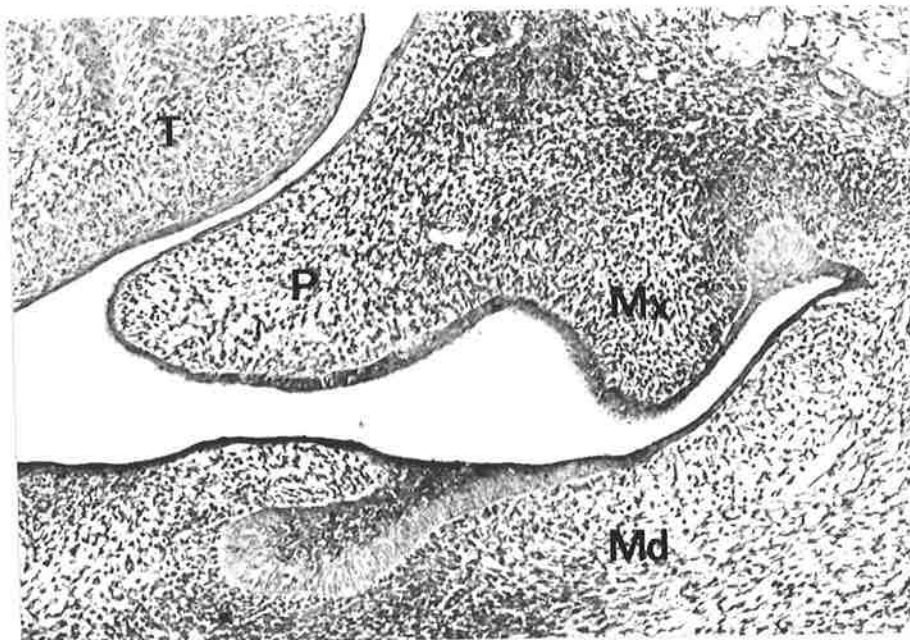
Fig.25 Tooth germs at bud stage. Dental development in the
mandible(Md) is more advanced than in the maxilla(Mx).
Tongue(T), Palatal shelf(P). (PAS, x100)
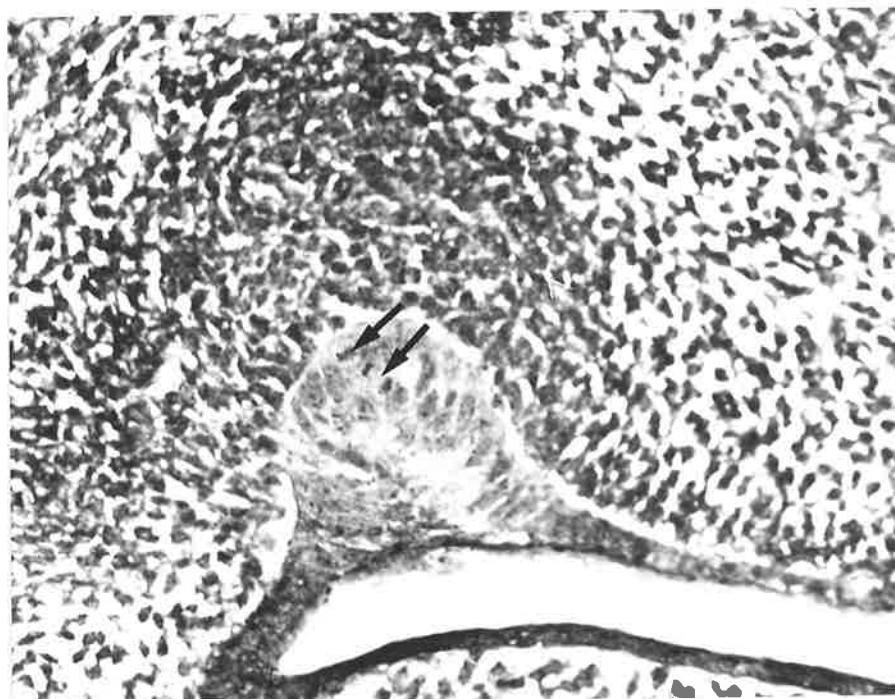
Fig.26 Dental lamina at the maxillary first molar regions.
Mitotic figures(arrows) are evident.
(PAS, x312).

14½ day old embyros

At this stage, the structures in the developing facial complex were seen to be in an advanced stage of development(Fig. 27). Both Meckel's cartilage and the septal cartilages were darkly staining due to their high mucopolysaccharide content. The musculature of the tongue was clearly differentiated although immature. Within the mandibular process, spicules of developing bone(of the woven variety) could be seen (Fig. 28).

The dental lamina had continued its inward growth into the mesodermal tissues. Invaginations were seen on the distal aspects of the mandibular tooth buds, the progenitors of the cap stage(Fig. 29). The cells of the mesoderm were deeply staining and markedly oriented around the dental primodia.

The tooth buds of the maxillary first molars were now in the bud stage of development, connected to the dental lamina by a short, thick stalk. There was a definite increase in the degree of epithelial proliferation over the 13½ day tooth bud. The cells surrounding the enamel organ were organised in the form of a whorl-like ball and were more closely packed than those found elsewhere. There was a marked decrease in the amount of intercellular spaces and processes between these cells. Numerous deeply stained cells appeared scattered through out the stroma of the mesoderm.

Fig.27
More advanced development
of the facial structures
is seen at 14½ days post-
conception.
(PAS, x30)
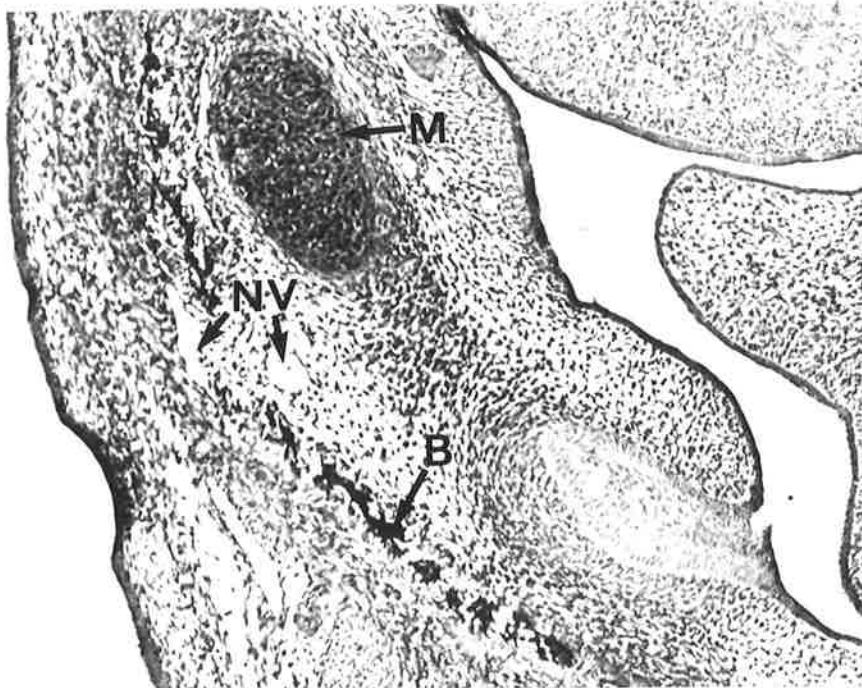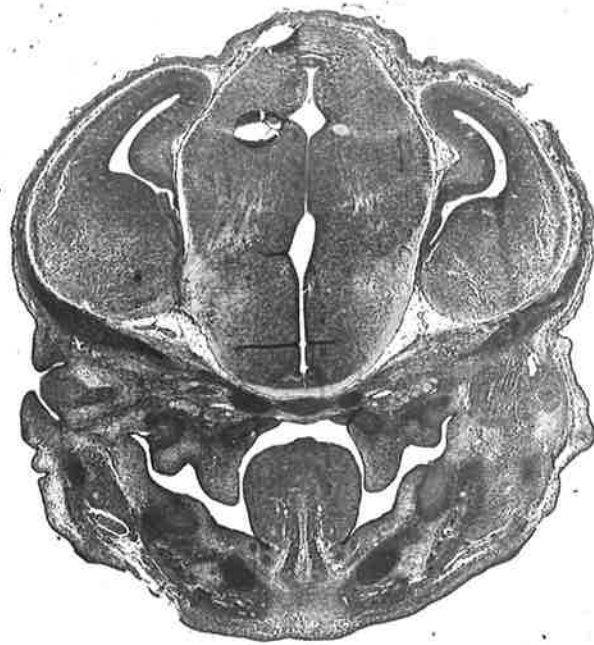
Fig.28 Meckel's cartilage(M), neuro-vascular bundle(NV) and
ossifying bone(B) seen within the mandible.
(PAS, x80)
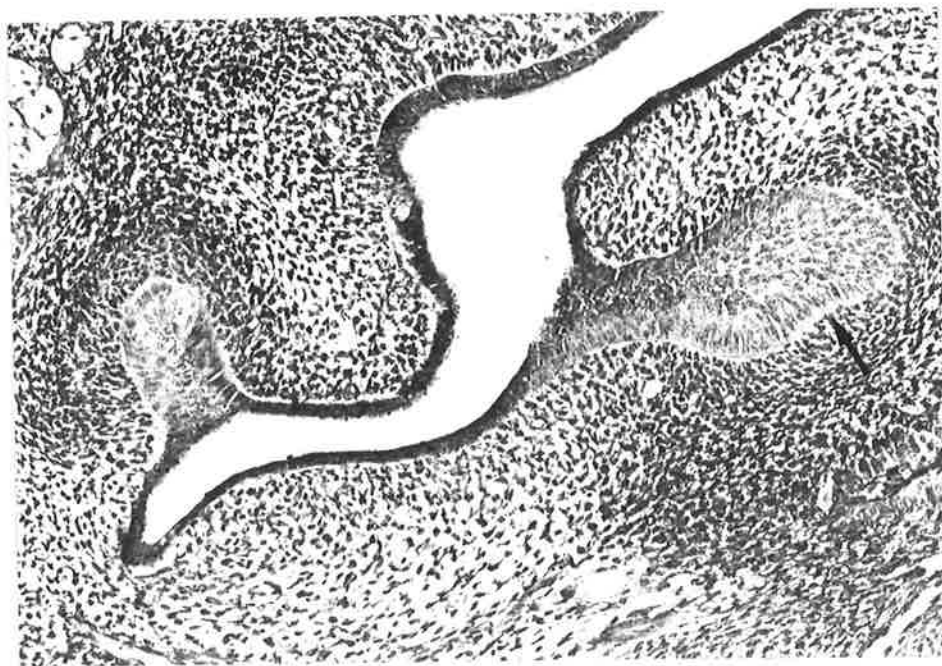
Fig.29 First indication of cap stage(arrow) development in the
mandibular tooth germ. Note heavy condensation of PAS
positive cells around the tooth buds.
(PAS, x75)

PART 2. TRANSMISSION ELECTRON MICROSCOPY

The maxillary molar anlagen, embedded in plastic, were first examined under the light microscope to allow an overall comprehension of the tissue topography.

12½ day old embryos

Figure 30 shows a primary epithelial band within 12½ day old embryos. At this stage, no ingrowth of the epithelium has occurred, but an initial thickening was visibly present. At the thickest portion, the cells were seen to be about 5 layers, the most superficial ones being squamoid in shape. The cells in the deeper stratum were essentially cuboidal, with their nuclei occupying nearly the whole of the cell. It was not uncommon to observe more than one nucleolus within a particular nucleus.

The basal layer of cells was seen to be low columnar in shape, with the basal membrane underneath it. At this stage, there was an ill-defined and diffused arrangement of the mesodermal cells underneath the basal membrane. There was no evidence of histodifferentiation; instead, the cells appeared to be immature and uniformly stained. They were polyhedral in shape and often loosely connected to one another by means of intercellular processes. There was also little alteration in the staining characteristics of the extracellular areas, and at the interface of the basal membrane, the cells of the mesenchyme appeared to be separated from the epithelium by a clear zone.

Further away, but still within dental mesenchyme, numerous blood capillaries were seen; many of them collapsed but some still patent and bearing immature blood cells. The relationship of the thickened oral
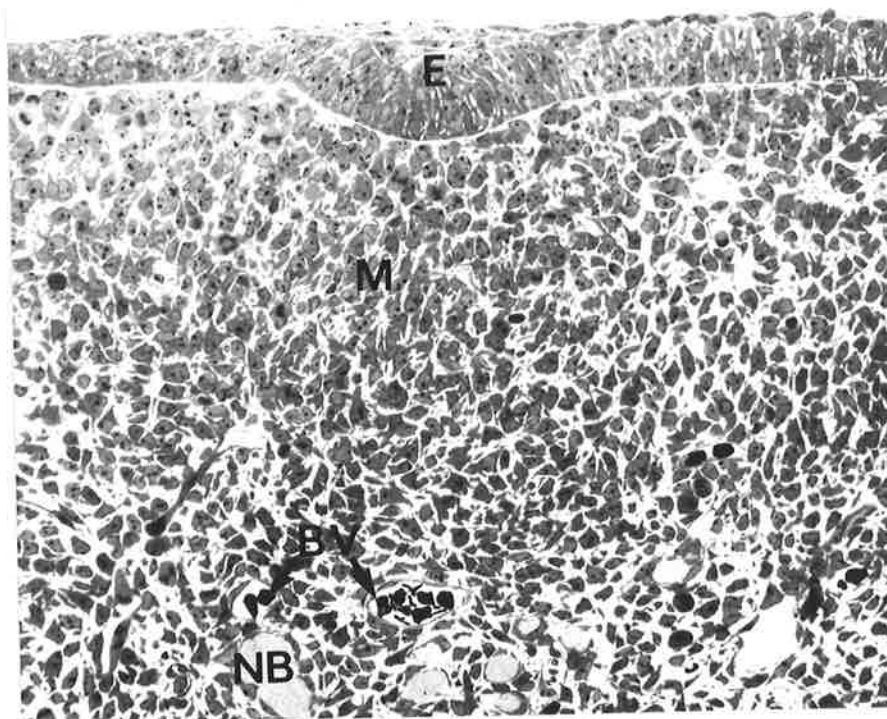
Fig.30 Oral epithelium(E) has thickened to form the dental lamina
primordium. Underlying mesenchyme(M) remain unorganized but
infiltrated by numerous blood vessels(BV) and nerve bundles
(NB).

12½ days postconception. EPON section. (Toluidine Blue, x200)

epithelium to the neuro-vascular elements was further reinforced by the observation of larger blood vessels(and nerve bundles) a little further away.

ULTRASTRUCTURE

The survey electron micrograph(Fig. 31) conveyed an overall impression of tissue immaturity; reflected by uniformly stained cells within the mesenchyme, polyhedral in shape and with relatively large nuclei. The epithelial-mesenchymal interface was marked by an intact basal lamina; underneath it were numerous mesenchymal cell processes and matrix fibrils. The fibrils were for the most part attached to the undersurface of the basal lamina(lamina densa) but were also intimately associated with the cell processes and cell membranes of the mesenchyme(Fig. 32).

The extracellular matrix(ECM) content of the basal lamina region(Fig.33) was better visualized in tissues fixed with tannic acid-glutaraldehyde. It was seen to be made up of web-like strands and blob-like material, insinuated in between adjacent cells and the basal lamina. Numerous cell processes and matrix fibrils were also prominently featured within this diffused stroma and the cell membranes closest to the basal lamina were also settled with this electron-dense precipitate. No banding was detectable within the matrix fibrils found in this area.

Further away, the intercellular areas of the mesenchyme were conspicuously empty of electron-dense ECM although occasionally, darker staining fibres resembling collagen appeared to be secreted by the cell surfaces(Fig. 34). The cytoplasm was characteristically undifferentiated, with plentiful ribosomes and mitochondria but sparse in Golgi apparatus and

endoplasmic reticulum. Occasionally, a microvesicular sac could be seen protruding from the cell membrane, each sac encapsulating a number of smaller vesicles.

At regions furthest away from the epithelium, the features of the mesenchyme remained basically unchanged, except for the presence of blood vessels(Fig. 35). The lumen of the vascular endothelia was occupied by still immature blood cells carrying prominent nuclei. Surrounding the blood capillaries were mesenchymal cells in their undifferentiated state; but interestingly, small quantities of glycogen were sometimes detectable within their cytoplasm. The intimate relationship between glycogen-containing cells and vascular endothelia was a consistent feature of the mesenchyme in these regions(Figs. 36, 37). A nerve bundle(Fig. 38), made up by numerous axons in a fascicular arrangement and undergoing initial myelination, could also be seen in the area.
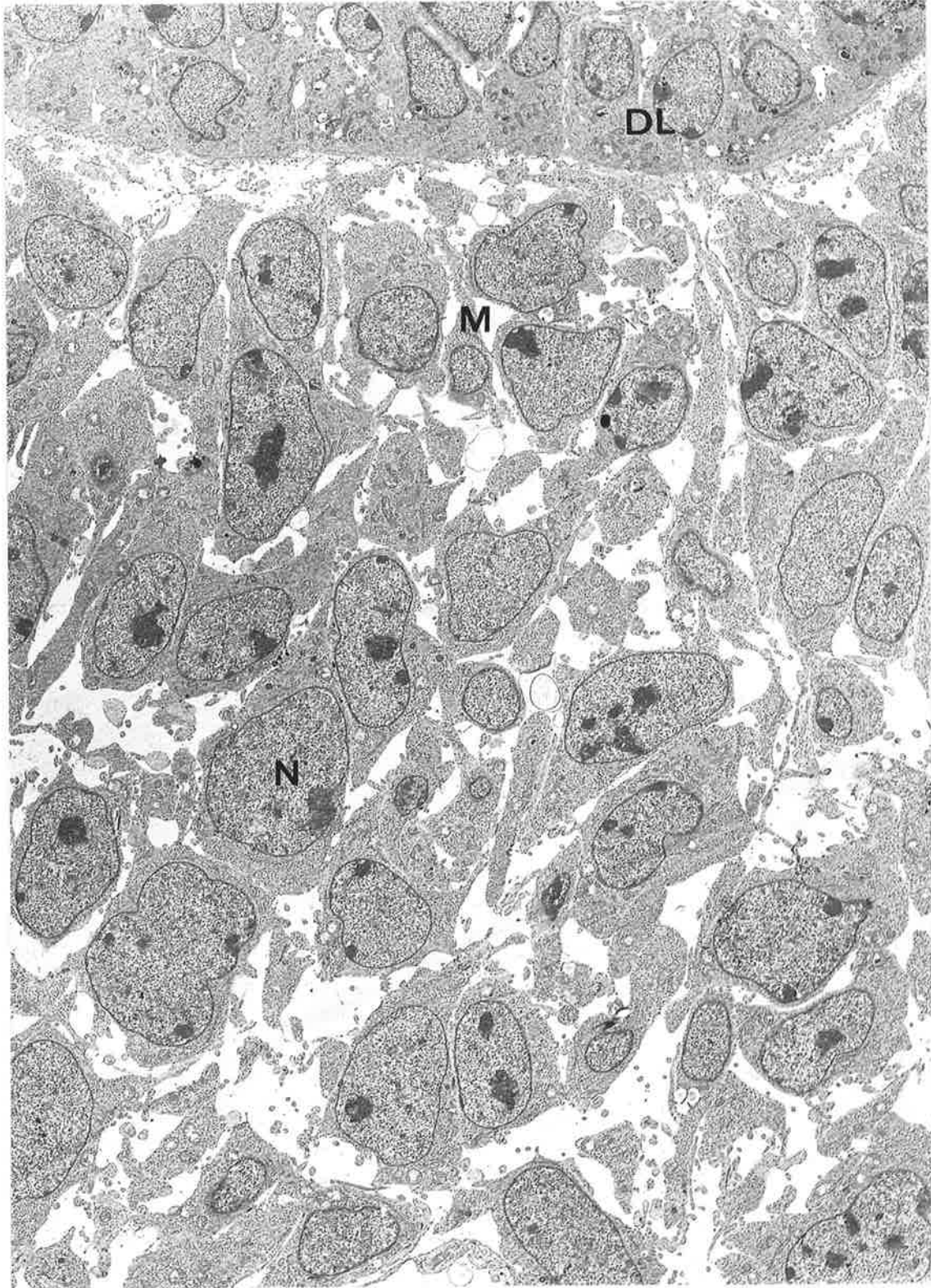
Fig.31 Survey electron micrograph of dental lamina primordium(DL)
and adjacent mesenchyme(M). The mesenchymal cells have large
nuclei(N) but no glycogen cells were detectable at this stage.
12½ days postconception.   ×2000
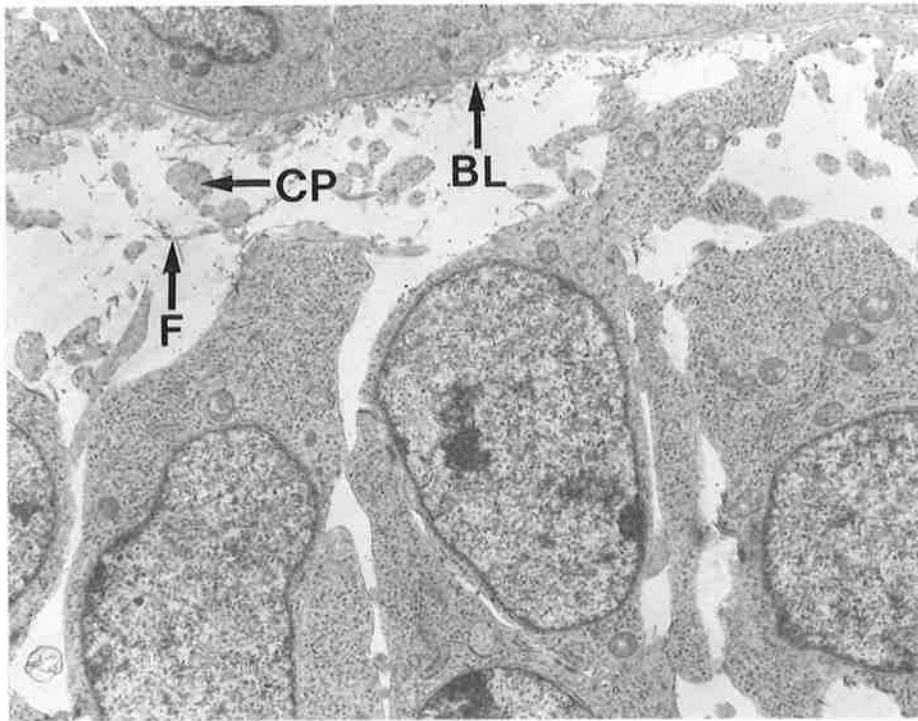
Fig.32 The basal lamina(BL) region was plentiful in matrix fibrils(F)
and cell processes(CP).
12½ days postconception. x7000
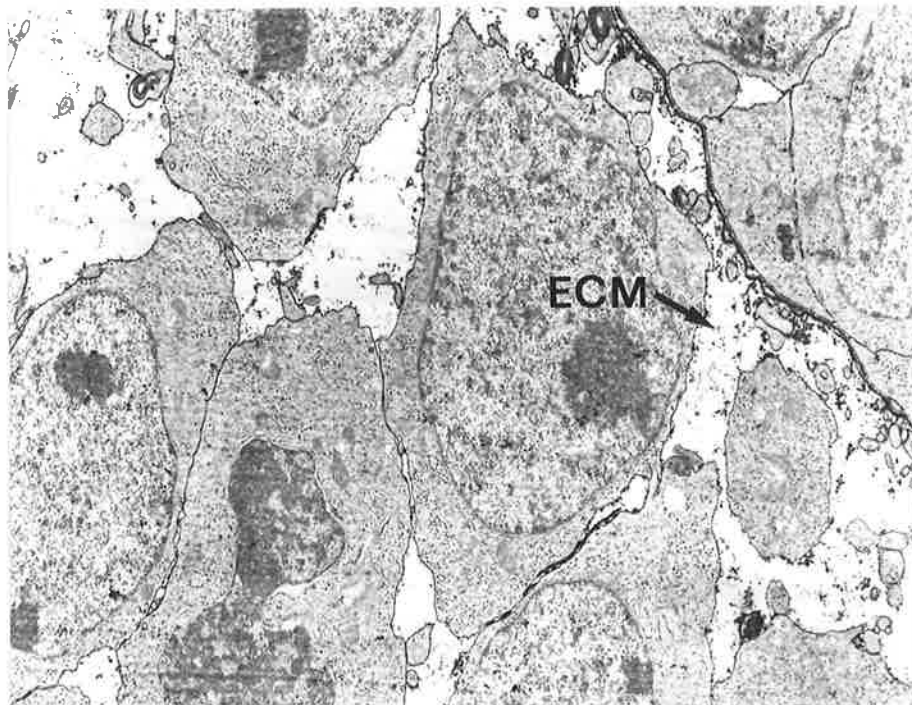
Fig.33 With Tannic acid-glutaraldehyde fixation, an electron-dense
precipitate(ECM) was found on the undersurface of the basal
lamina.
12½ days postconception. x7000
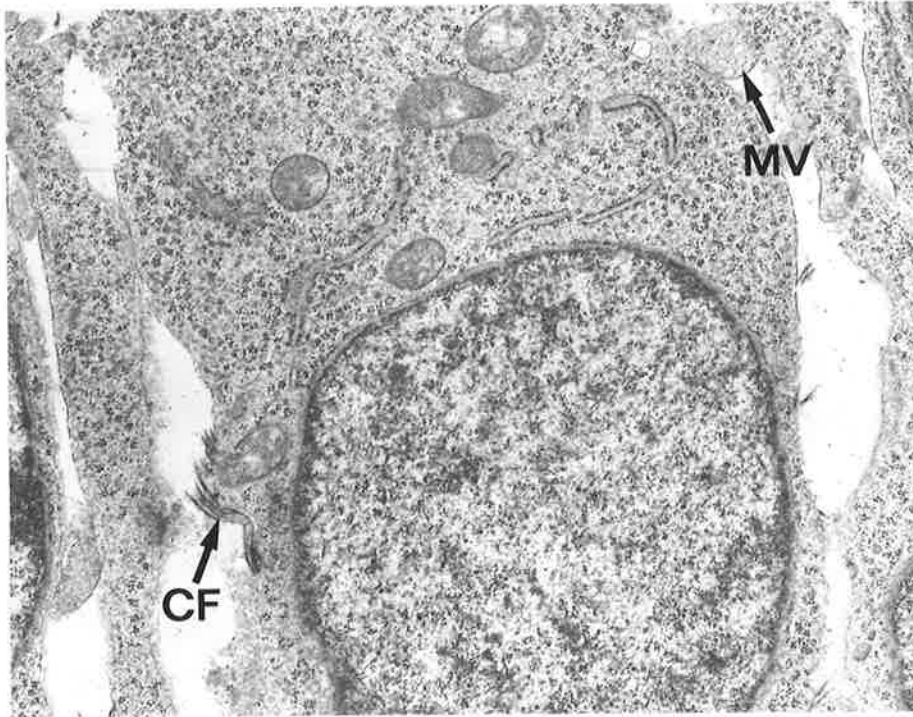
Fig.34 Banded collagen fibrils(CF) intimately related to the cell
surface of embryonic dental mesenchyme. A microvesicular sac
(MV) can be seen protruding from the cell membrane.
12½ days postconception. x25,000
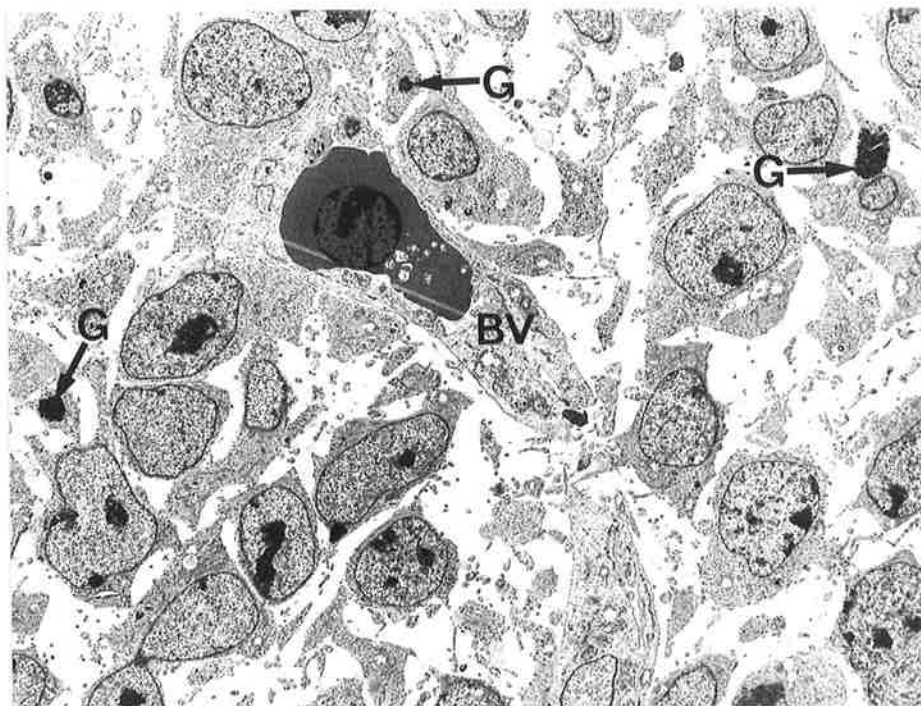
Fig.35 At regions further away from the dental lamina primordium,
the blood vessel(BV) is often found close to mesenchyme
carrying small quantities of glycogen particles(G).
12½ days postconception. x2,000
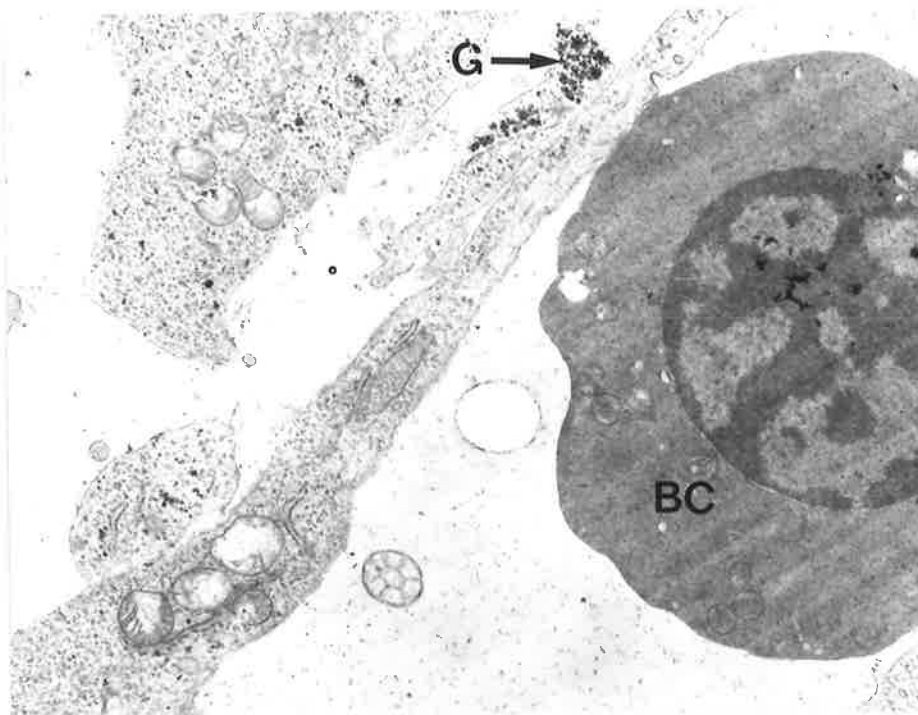
Fig.36 Immature blood cell(BC) found within the lumen of vascular
endothelia closely related to glycogen-containing(G) cell
process. 12½ days postconception. x7,000
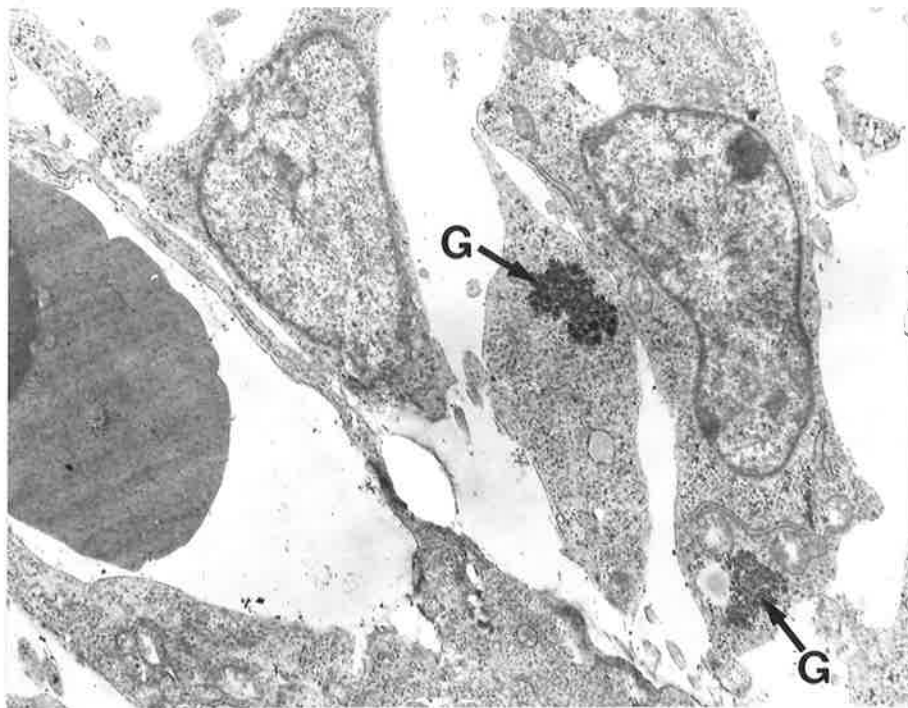
Fig.37 Another instance of glycogen(G) presence within cytoplasm
of perivascular mesenchyme in areas distant to the dental
lamina primordium.
12½ days postconception. x6,000
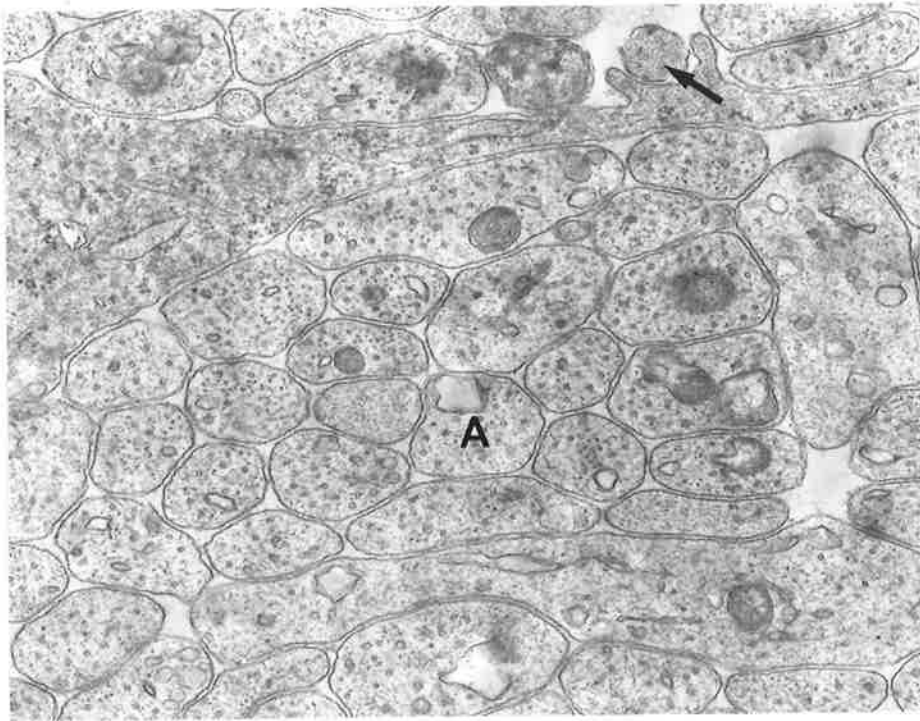
Fig.38 Nerve bundle with cut axons(A). An immature Schwann cell
is seen encircling a nerve fibre(arrow).
12½ days postconception. x16,000

<u>13½ day old embryos</u>

At 13½ days, the dental lamina had formed as an epithelial ingrowth
into the maxillary process mesenchyme(Fig. 39). The cells within the
dental lamina were cuboidal in shape, darkly staining and closely packed
except for those in the apical areas that remained stellate and loosely
arranged. Underneath the basement membrane and closely apposed to this
structure was a well-marked area of mesenchymal condensation. The cells
were intensely stained and closely packed into a whorl-like arrangement.
Numerous blood capillaries could be seen insinuated within this cell mass.
Further away, the mesenchyme consisted of loosely arranged cells;
separated by large intercellular spaces and devoid of any structural
orientation.

<u>ULTRASTRUCTURE</u>

The most striking feature of the mesenchyme, when compared with
that in 12½ day embryos, was the widespread occurrence of glycogen
within the cell cytoplasm(Figs. 40-44). The glycogen granules were mostly
rosettes(alpha particles, 100 nm diameter) aggregated together to form
a large clump to the exclusion of other cellular organelles(Figs. 41,
42, 44). At other areas, the glycogen particles could be found lined
against the membranous walls of empty vesicles. Single glycogen particles
in the beta form(15-40 nm in diameter) were mostly strewn within the
cytoplasm in a diffused fashion(Fig. 42). No active secretion of the
glycogen material into the extracellular matrix(ECM) was detectable
although residual glycogen particles were sometimes found within the lumen
of large superficial microvesicular sacs(Fig. 41). Although frequently
lined against the external walls of the endoplasmic reticulum, glycogen
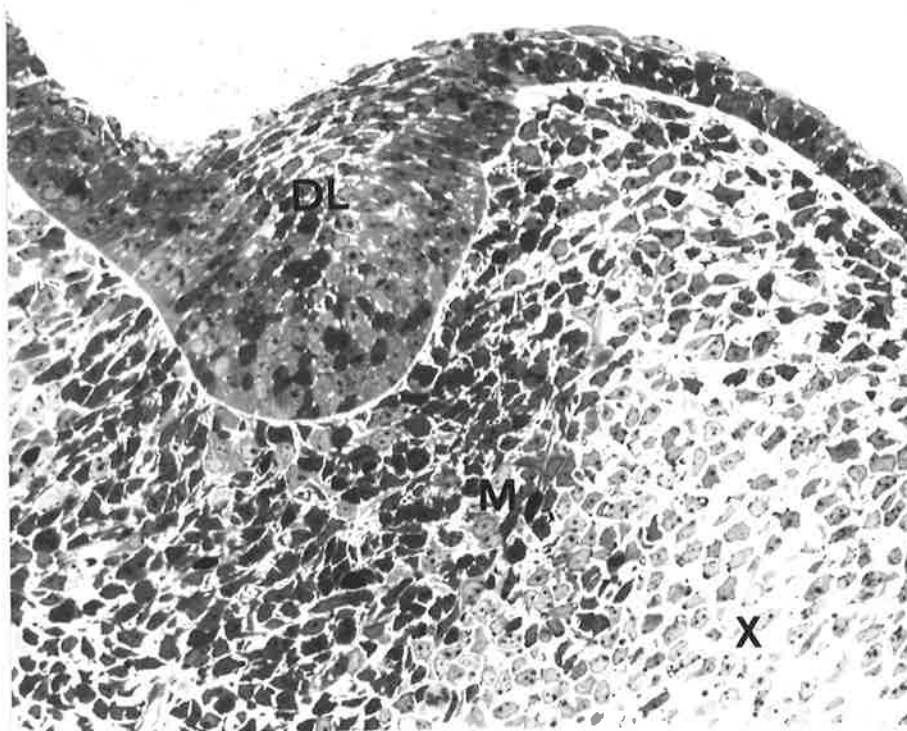
Fig.39 Dental lamina(DL) seen as an epithelial ingrowth into the
underlying mesenchyme(M) which is darkly staining and
arranged in a whorl-like manner.
The mesenchyme in distal regions(X) are lightly stained
and have large and empty intercellular spaces.
13½ days postconception. x250
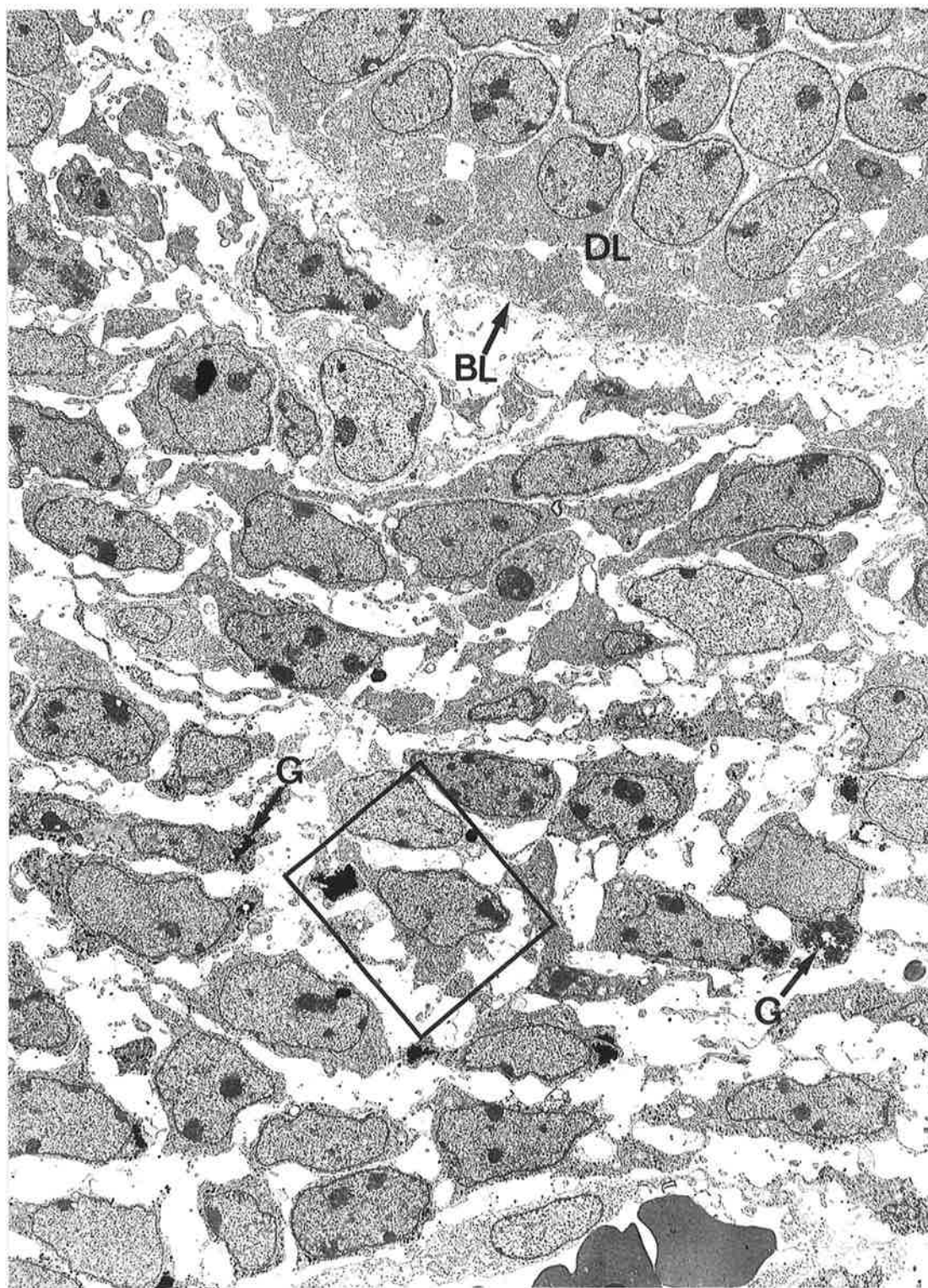
Fig.40 Survey electron micrograph showing stellate-shaped dental
mesenchyme adjacent to dental lamina(DL). Darkly stained
glycogen granules(G) can be seen within cytoplasm but only
in mesenchyme four to five cells away from the basal lamina
(BL). Note increase in amount of ECM material underneath the
basal lamina.
$13\frac{1}{2}$ days postconception. x3,000
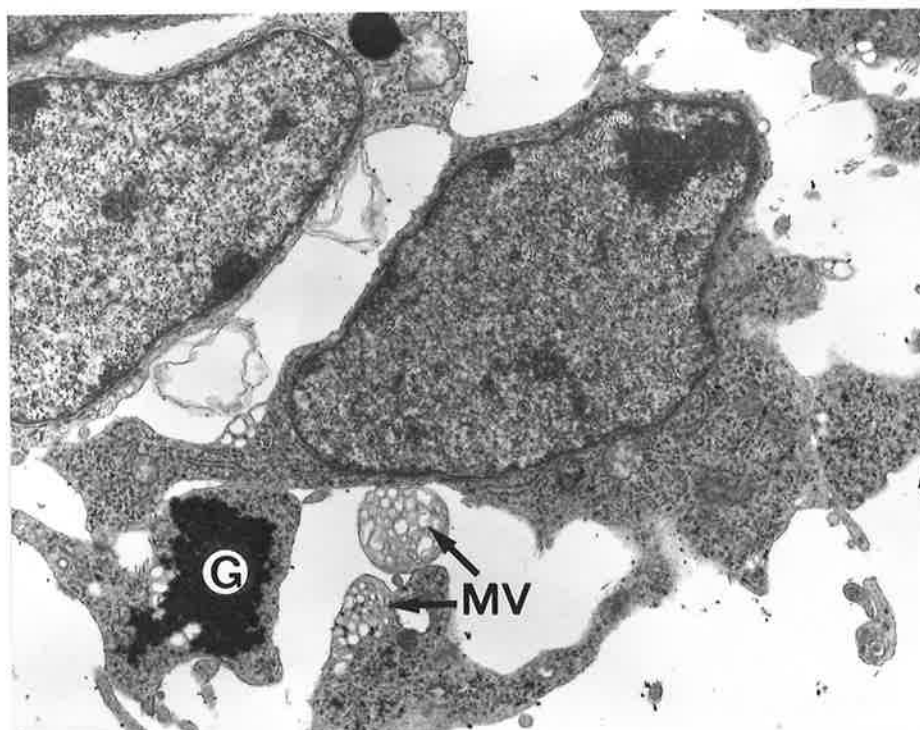
Fig.41 Typical cell boxed in Figure 40. Microvesicular sacs(MV) and glycogen(G) were both consistent features. Occasionally, glycogen particles could be found within the lumen of micro- vesicular sacs. 13½ days postconception. x10,000
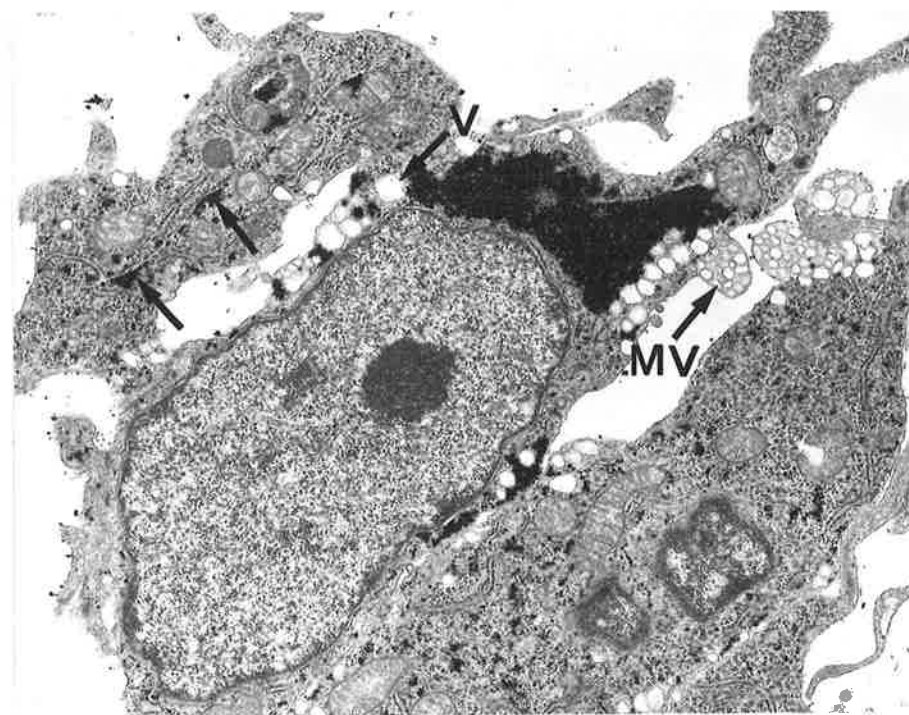
Fig.42 Another glycogen-rich cell with large intracellular vesicles (V) and microvesicular sacs(MV). Glycogen particles were sometimes found loosely strewn within cytoplasm; at other times, lined against endoplasmic reticulum(arrows). 13½ days postconception. x10,000
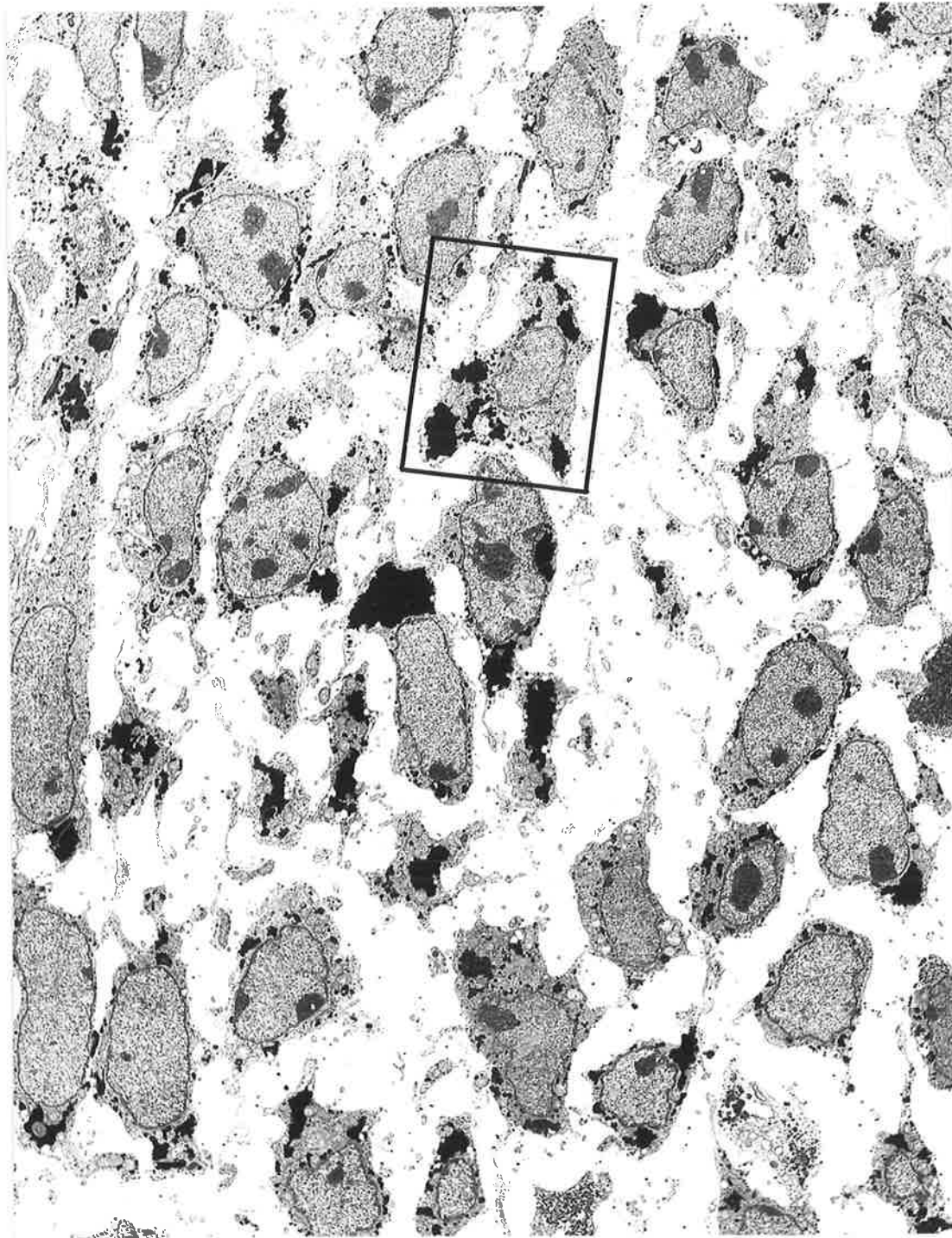
Fig.43 Survey electron micrograph of dental mesenchyme found
further away from the epithelial dental lamina. Note
increased quantities of intracellular glycogen and large
extracellular spaces occupied by cellular processes
(filopodia).
13½ days postconception. x2,000
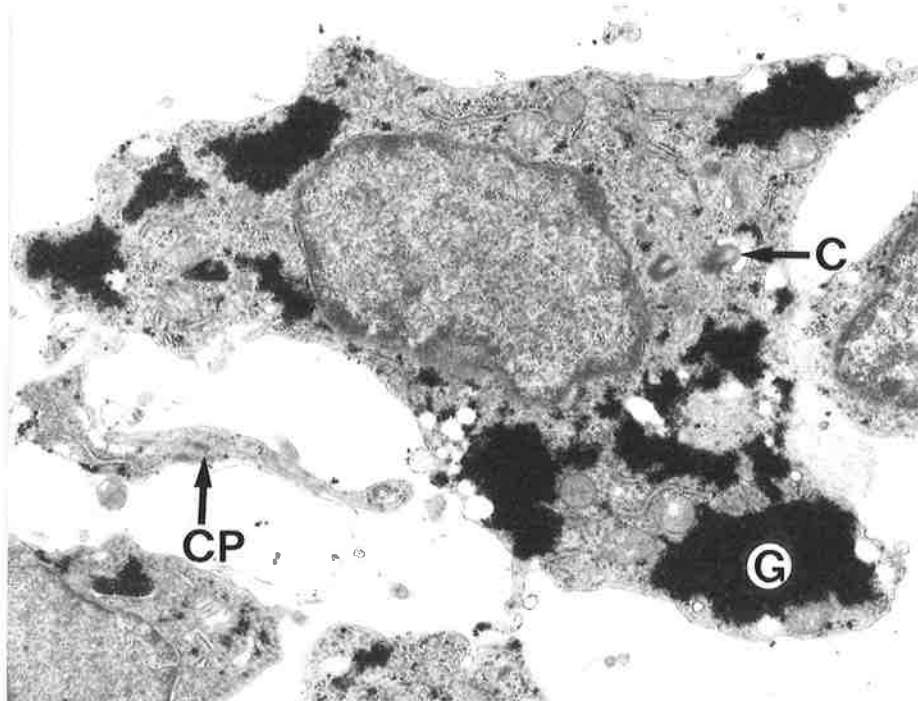
Fig.44 A typical mesenchymal cell found boxed in Figure 43.
Glycogen(G) granules occupy the cytoplasm almost to the
exclusion of other cellular organelles.
Cilium(C), Filopodia(CP).
$13\frac{1}{2}$ days postconception. x9,000

was at no time found within the cisternae itself(Fig. 42).

A comparison of the glycogen content within the mesenchyme proximal
and distal to the dental lamina revealed significant regional differences.
The cells proximal to the dental epithelium had a diminished quantity
of glycogen(Fig. 40) - both in content and distribution - when compared
to the cells found in the distal areas of the mesenchyme(Fig. 43).
This difference, in the character of the mesenchyme in proximal and distal
areas, was also reflected in the extracellular matrix(ECM). After fixation
with tannic acid-glutaraldehyde, the tissues revealed a higher concentra-
tion of ECM among the distal mesenchyme, whereas the intercellular spaces
of the proximal mesenchyme were relatively empty(Fig. 45). At higher
magnification, the ECM of the distal mesenchyme was seen to consist of
thread-like material(2nm in diameter, 6 nm in length), possibly hyaluronic
acid; with electron-dense granules(possibly proteoglycans) on them. They
were found, either free in the intercellular regions or linked to cell
surfaces (Fig. 46) and where a filopodia was present, it was invariably
the focal point of attachment for the matrix material resulting in a
radial outgrowth(Fig. 47). Another prominent feature of the ECM was the
presence of banded collagen fibrils in close association with the
thread-like material and cellular surfaces, sometimes forming a link
between the two components(Figs. 48, 49).

The other area richly possessed of ECM was the undersurface of the
basal lamina. The picture remained essentially unchanged from that observed
in 12½ day tissues; but there could have been a quantitative increase in
matrix fibrils and electron-dense precipitate(Fig. 50).
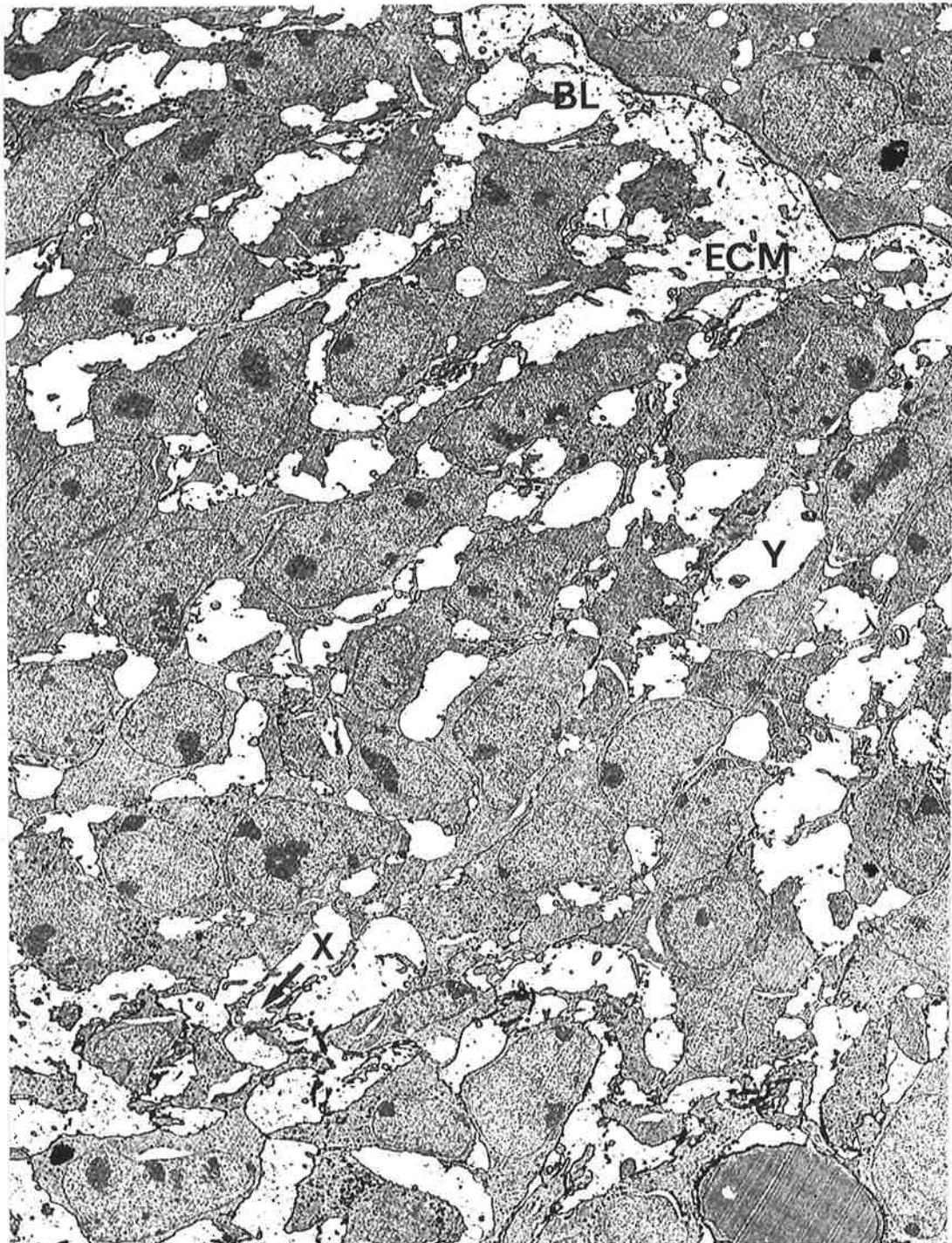
Fig.45 Fixation with Tannic acid-glutaraldehyde.
Note increase in ECM content within distal mesenchyme(X)
when compared to relatively empty intercellular areas
in proximal mesenchyme(Y). However, the basal lamina(BL)
region is still heavily concentrated with ECM material.
13½ days postconception. x2,000
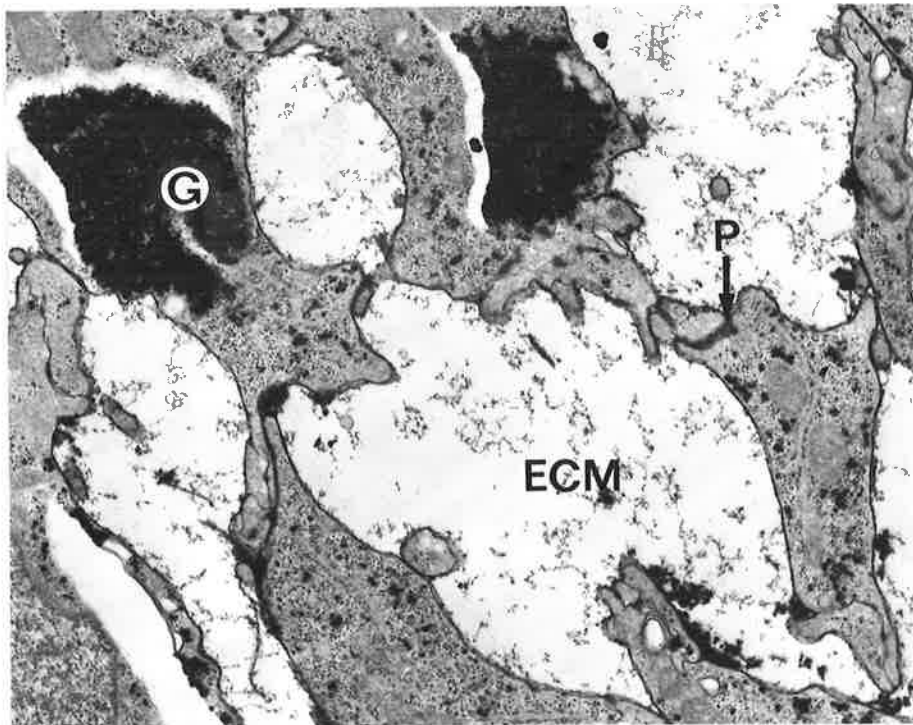
Fig.46 Tannic acid-glutaraldehyde fixation.
Distal mesenchyme linked to each other by means of electron-
dense plaques(P). Note heavy concentration of ECM material.
Glycogen(G). 13½ days postconception. x20,000
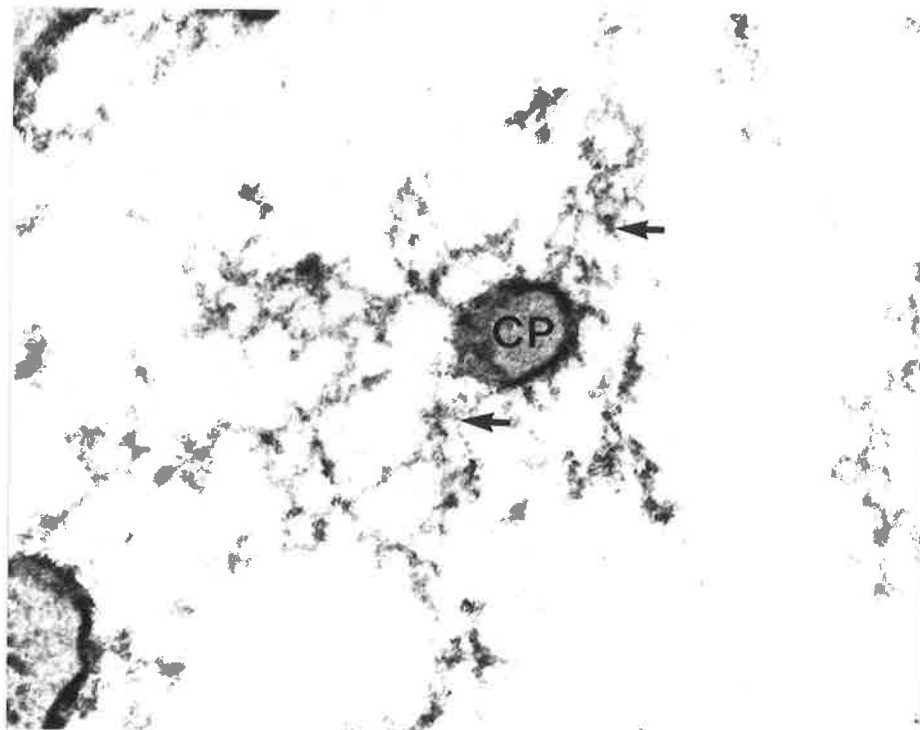
Fig.47 Tannic acid-glutaraldehyde fixation.
Single filopodium(CP) becoming the focal point of attach-
ment for thread-like material(arrows) carrying darker
staining blobs. 13½ days postconception. x60,000
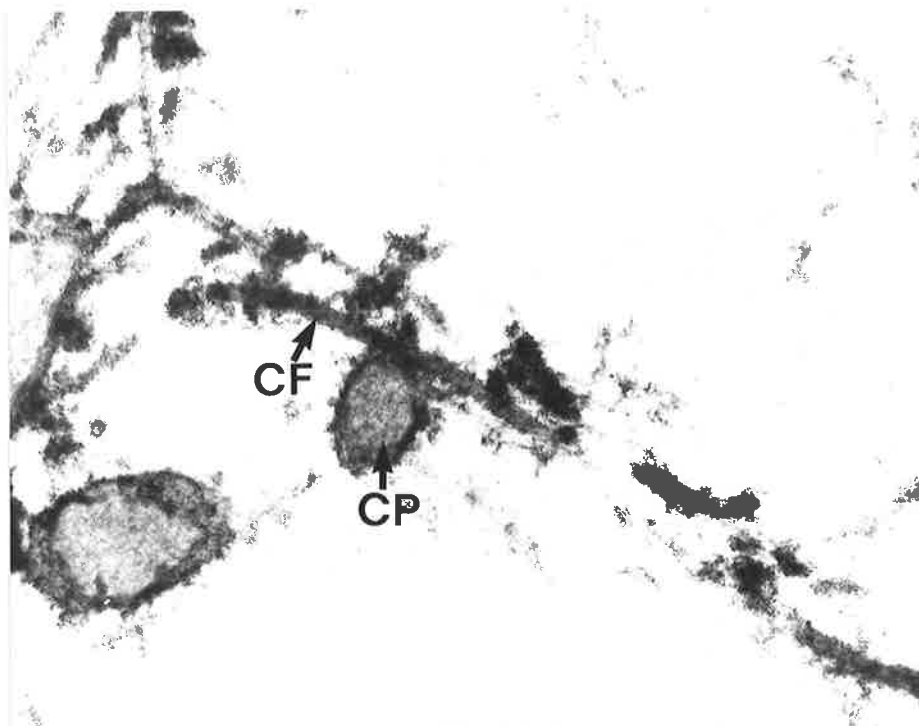
Fig.48 Tannic acid-glutaraldehyde fixation.
Banded collagen fibrils(CF) found in close association with
filopodium(CP) and ECM material.
13½ days postconception. x70,000
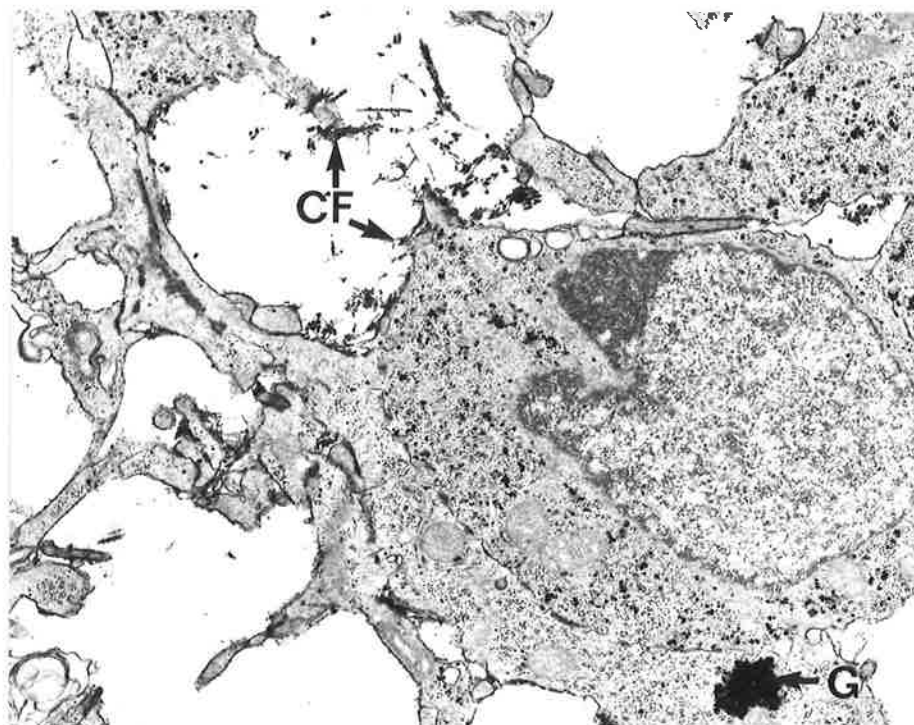
Fig.49 Tannic acid-glutaraldehyde fixation.
Collagen fibrils(CF) and electron-dense ECM found close to
cellular processes and cell membranes.
Glycogen(G). 13½ days postconception. x10,000
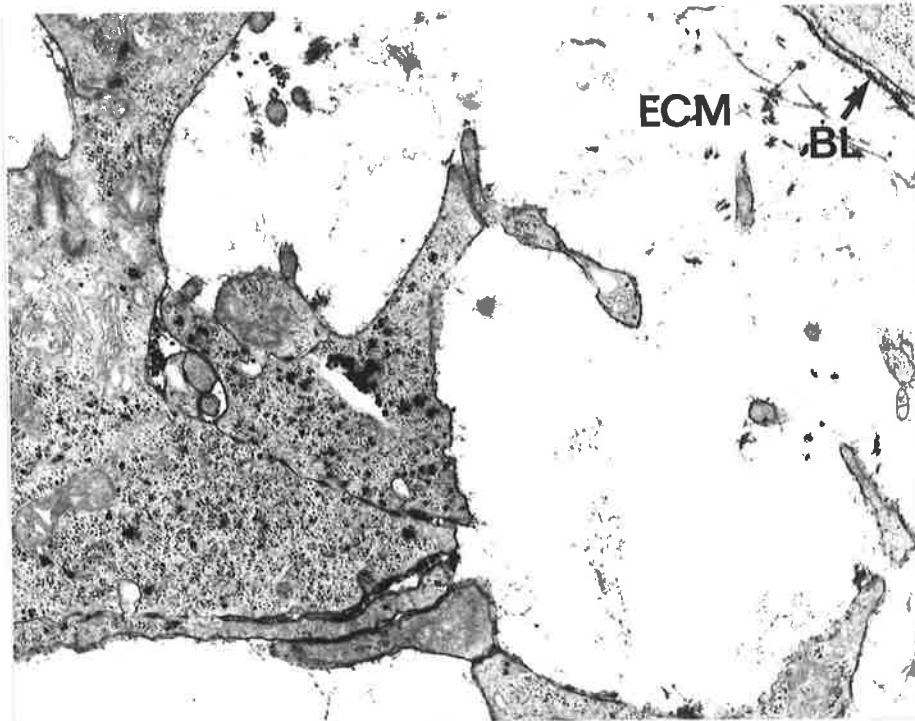
Fig.50 Tannic acid-glutaraldehyde fixation.
Rich ECM material found close to basal lamina(BL).
13½ days postconception. x12,000
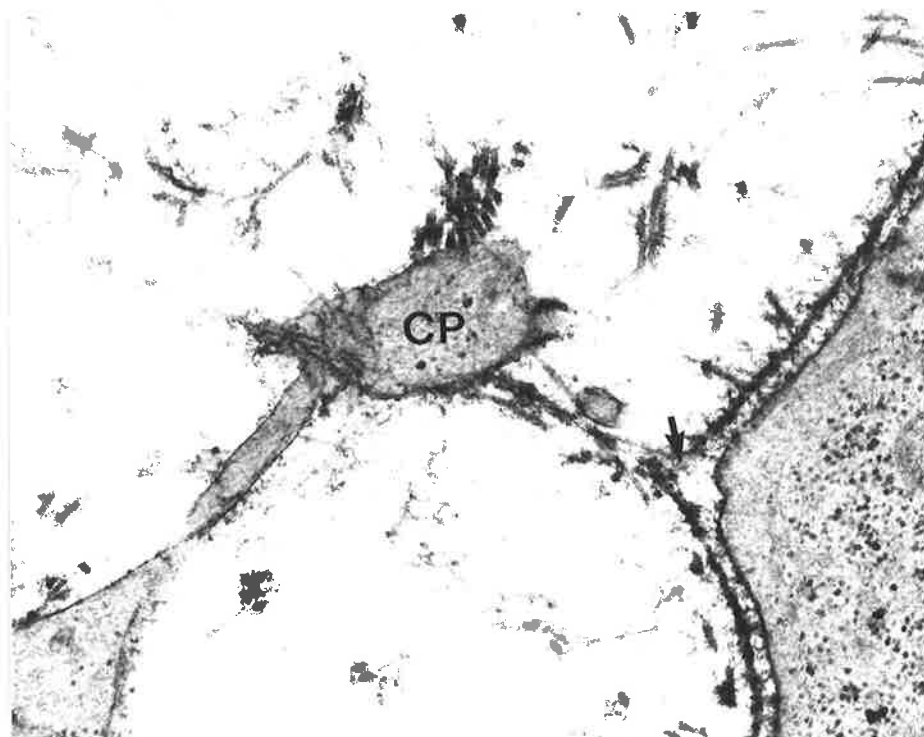
Fig.51 Tannic acid-glutaraldehyde fixation.
Disruption of basal lamina(arrow) with collagen fibrils lying
in its path and linked to mesenchymal cell process(CP).
13½ days postconception. x30,000
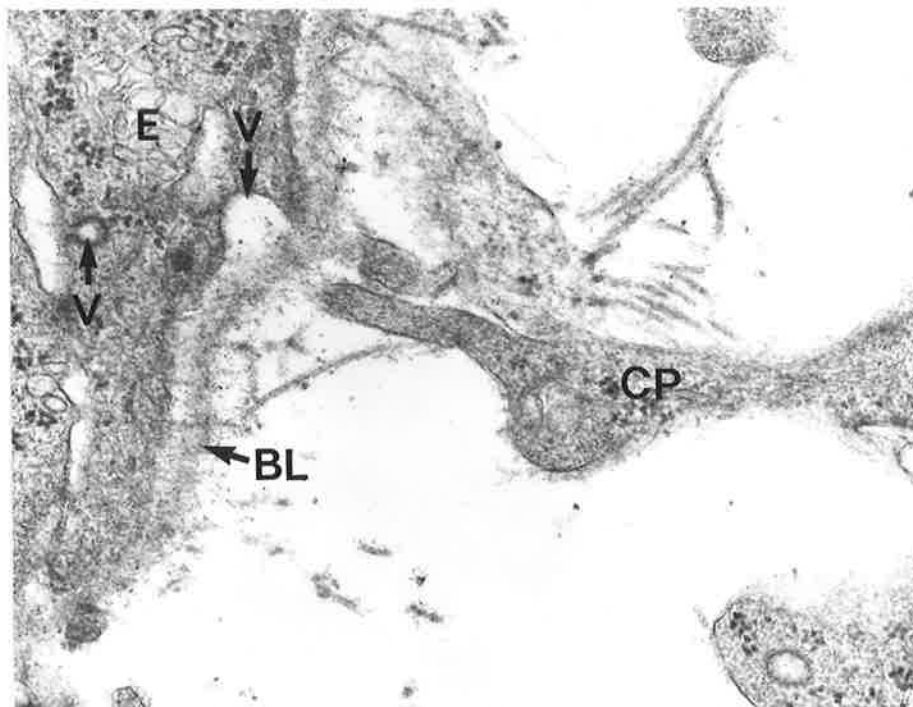
Fig.52 Intact basal lamina(BL) interposed between mesenchymal cell
processes(CP) and epithelium(E). Note patent vesicles(V)
within epithelial cell.
13½ days postconception. x40,000
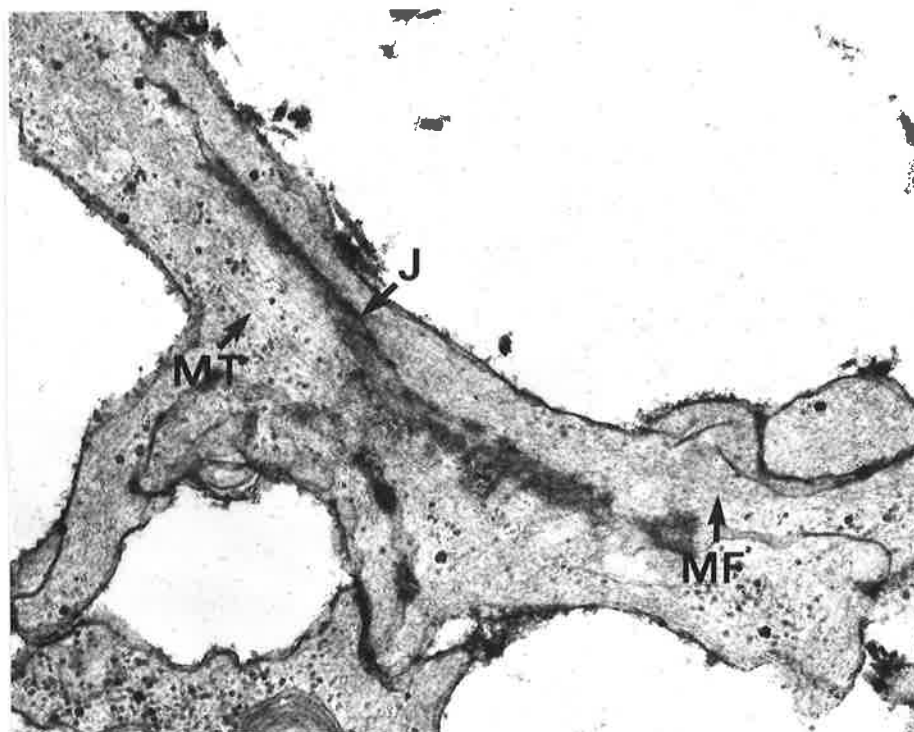
Fig.53 Tannic acid-glutaraldehyde fixation.
Microfilaments(MF) seen running into electron-dense cell
junction(J) between adjacent filopodia. A microtubule(MT)
can be seen close by.
13½ days postconception. x30,000

Upon closer examination of the basal lamina underneath the dental epithelium, a localised disruption of the otherwise intact lamina densa could be seen and banded collagenous material found lying in its path straddled in between epithelial and mesenchymal cells(Fig. 51). At other areas, the filopodia of mesenchyme were found directly apposed to the undersurface of the basal lamina, above which were epithelial cells possessing vesicles in communication with the exterior(Fig. 52).

Apart from the glycogen content and ECM distribution, dental mesenchyme in 13½ day old embryos had other distinguishing features when compared with 12½ day old mesenchyme. The cells in 13½ day old mesenchyme were generally stellate in shape and had numerous filopodia; 12½ day old mesenchyme had more rounded cellular outlines. Where cells were in contact, the apposed cell membranes often formed electron-dense plaques reminiscent of epithelial tight junctions(Fig. 53). In addition, microfilaments(5-9 nm in diameter) were seen within the filopodia and inserted into the cell junctions(Figs. 53, 54). Occasionally, a microtubule approximately 20 nm in diameter could be seen(Fig. 53). Other organelles not exclusive of 13½ day old mesenchyme - in other words present in 12½ day old mesenchyme as well - but worthy of mention include microvesicular sacs(Fig. 42, 44) and cilia(Fig. 44, 55).
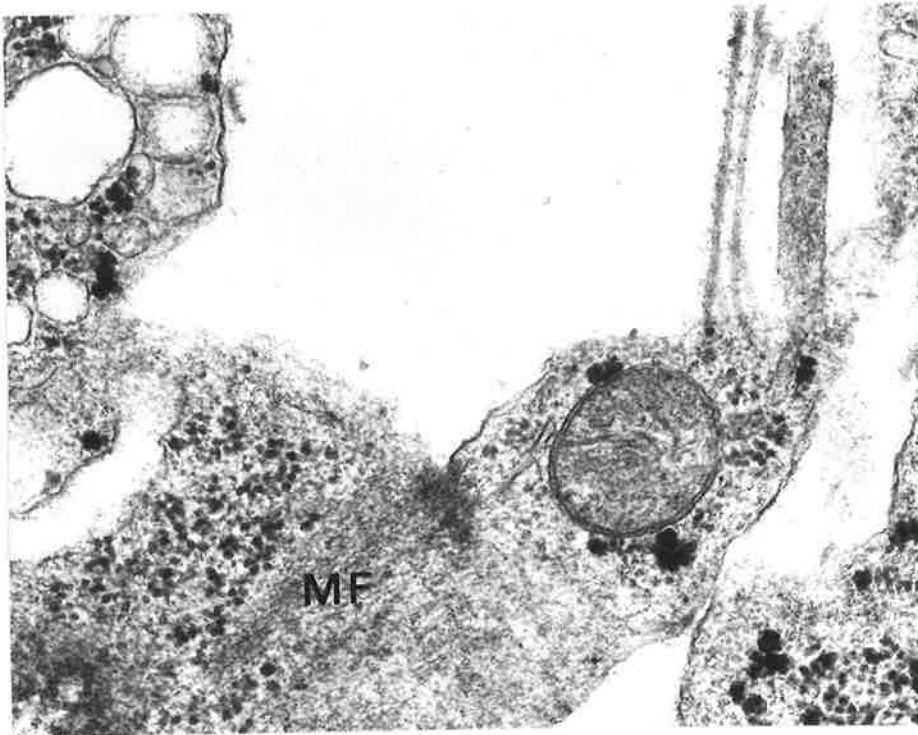
Fig.54 Microfilaments(MF) were regularly found within the cortical
regions of mesenchymal filopodia.
13½ days postconception. x40,000
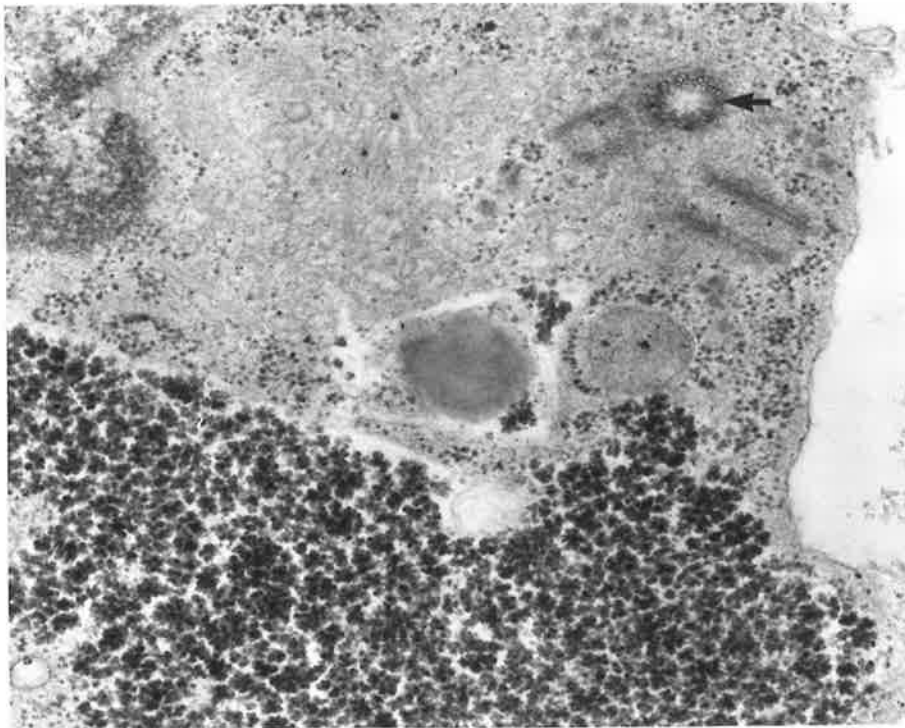
Fig.55 Cross-section of centriole(arrow) having a triplet arrange-
ment of microtubules.
13½ days postconception. x32,000

PART 3. ORGAN CULTURE

The age of the explants, at the time of culture, was confirmed by ultrastructural examination of zero-time controls using criteria developed in the previous section. The results of organ culture, judged by the presence or absence of teeth, are summarized in Table 4.

Table 4 - Results of organ culture

		12½ day embryos		13½ day embryos	
Culture period	Culture medium	No. of explants	No. forming teeth	No. of explants	No. forming teeth
7 days	M199*	4	0	4	4
10 days	M199*	4	0	4	3
15 days	M199*	4	0	4	2
15 days	BGJb**	12	0	2	2
16 days	BGJb**	-	-	2	2
17 days	BGJb**	-	-	2	1
18 days	BGJb**	-	-	2	1
19 days	BGJb**	-	-	2	2
20 days	BGJb**	-	-	1	1
30 days	BGJb**	-	-	1	0
	Total	24	0	24	18

*Study 1
**Study 2

The number of explants used for each age group(12½ and 13½ day embryos)
totalled 24, a dozen in Study 1(Medium 199) and a dozen in Study 2(BGJb
medium). Out of the 24 explants from 12½ day old embryos, none developed
teeth in organ culture. The number of days in culture was not prolonged
beyond 15 days, since it was felt to be pointless in this group, irrespective
of culture media.

As for explants from 13½ day old embryos, 18 out of 24 explants
produced tooth germs in varying stages of development, a success rate
of 75%. The explants were grown for varying periods of time, up to 30 days
in one case although it failed to produce teeth.

The developmental status of the recovered explants will be indivi-
dually described; with a representation from each batch of explants.

EXPLANTS FROM 12½ DAY OLD EMBRYOS

After 7 days *in vitro*(Fig. 56)

The explants remained healthy, with massive epithelial proliferation
and keratinization on their superior aspects. The basement membrane
appeared intact although poorly defined. The mesenchymal cells had lost
their orientation and were consist of a mixture of different cell shapes
and sizes. They appeared lightly stained, and loosely arranged within a
paler staining intercellular matrix. No blood vessels were seen but
cartilage was present.

There was no evidence of tooth formation.

After 10 days *in vitro*(Fig. 57)

There was no evidence of tooth formation but the epithelium had proliferated in a manner suggestive of a molar enamel organ. The mesenchymal cells remained disoriented and excessive keratin could be found on the epithelial surfaces.

After 15 days *in vitro*(Figs. 58, 59)

Explants were retrieved after culture in either Medium 199, or BGJb medium. In either case, no tooth germs were seen and instead, massive epithelial proliferation with attendant keratinization, attempting to surround a disoriented mesenchyme(Fig. 58). In some cases, epithelial encirclement appeared to be complete(Fig. 59) with many epithelial pearls trapped within the mesenchyme.
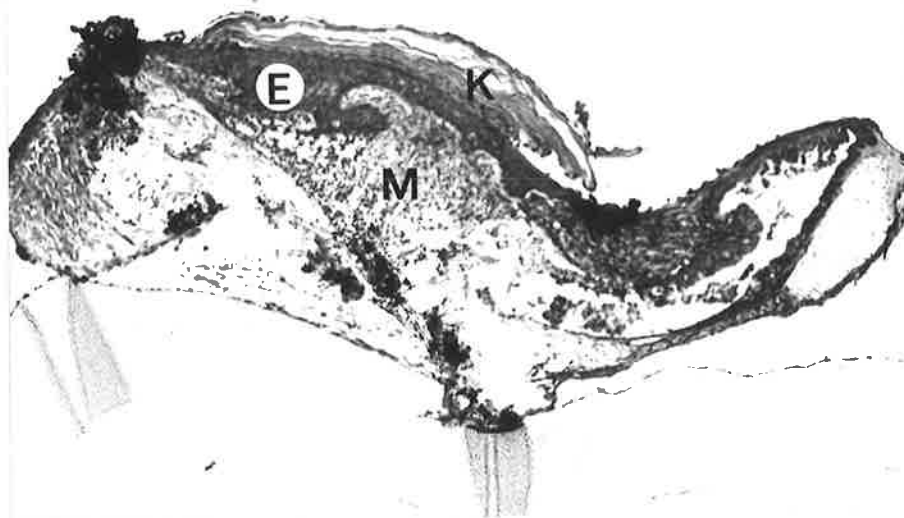
Fig.56 12½ day old explant, cultured for 7 days.
Disorganized epithelium(E) and mesenchyme(M) with keratin
formation on the epithelial surfaces.
(Haematoxylin & Eosin, x250)
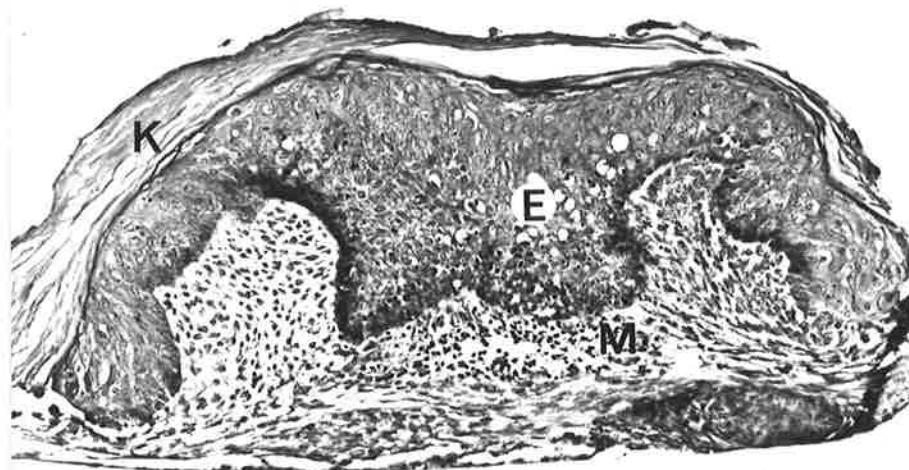
Fig.57 12½ day old explant, cultured for 10 days.
Epithelial proliferation in a manner suggestive of a molar
crown form but no dental elements were detectable.
Epithelium(E), Mesenchyme(M), Keratin(K).
(Haematoxylin & Eosin, x125)
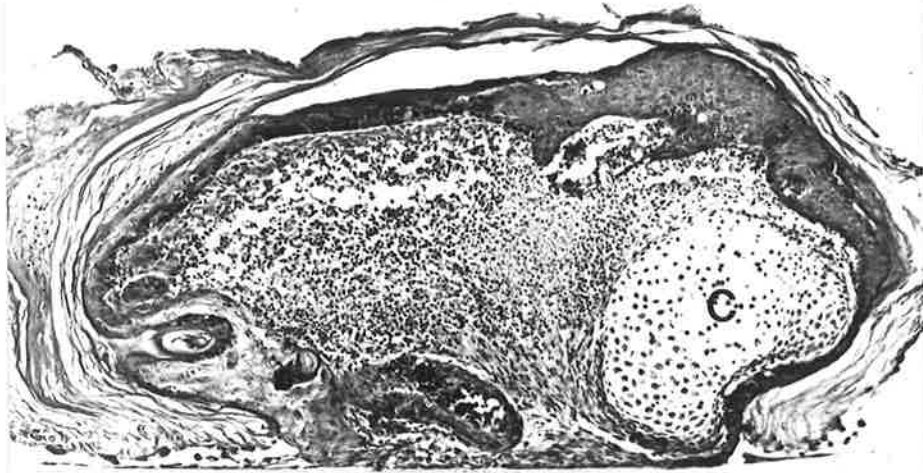
Fig.58 12½ day old explant, cultured for 15 days.
No evidence of tooth germ formation but cartilage(C) was
present. (Haematoxylin & Eosin, x100)
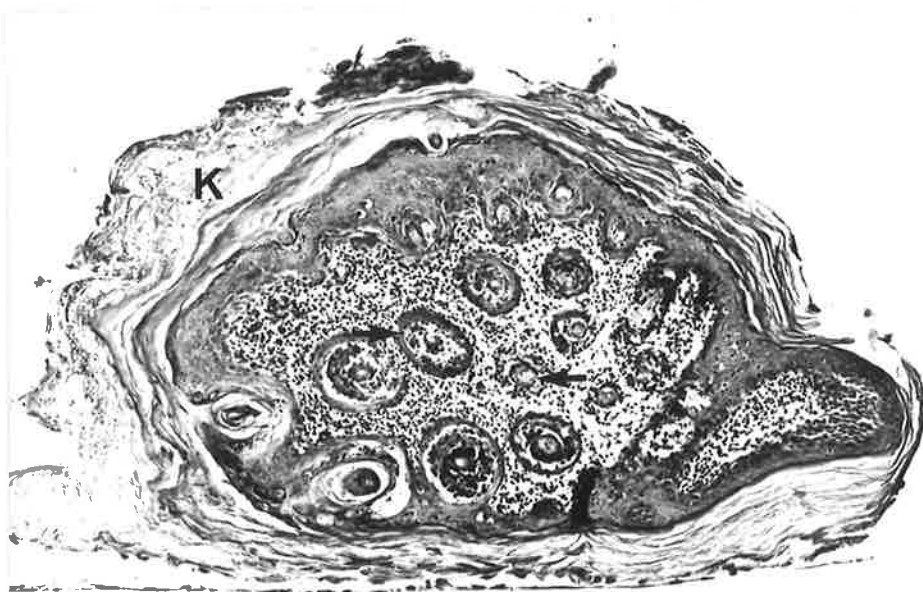
Fig.59 12½ day old explant, cultured for 15 days.
No evidence of tooth germ formation, instead massive epi-
thelial proliferation around and within disoriented mesen-
chyme. Keratin(K), Epithelial pearls(arrows).
(Haematoxylin & Eosin, x125)

EXPLANTS FROM 13½ DAY OLD EMBRYOS

After 7 days *in vitro* (Fig. 60)

The recovered explants consistently revealed the presence of an
enamel organ in the early bell-stage of development. The outer and inner
enamel epithelia were continuous at the cervical loop region. Within
the enamel organ, cells of the stellate reticulum and stratum intermedium
were found in a poorly stained matrix. The cells of the inner enamel
epithelium remained tall columnar with no evidence of nuclear polarization.
Next to this layer was a narrow eosinophilic zone, the site of the future
dento-enamel junction.

The cells of the dental papilla were mainly cuboidal, closely
packed together but with no evidence of odontoblast differentiation.
At the base of the dental papilla, the cells appeared to be organized
in the form of a primitive dental follicle, continuous with the remaining
parts of this ambient structure. Cartilage formation continued within the
explants and were often found in the immediate vicinity of the tooth germ,
a situation not normally found *in situ*.

After 10 days *in vitro* (Fig. 61)

The tooth germs were distinctively molariform and in the late bell
stage of development. The first indication of cusp formation was given
by the proliferating enamel epithelium at the centre; forming a spearhead
into the dental papilla. The cells of the inner enamel epithelium, the
future ameloblasts, remained undifferentiated with their nuclei scattered
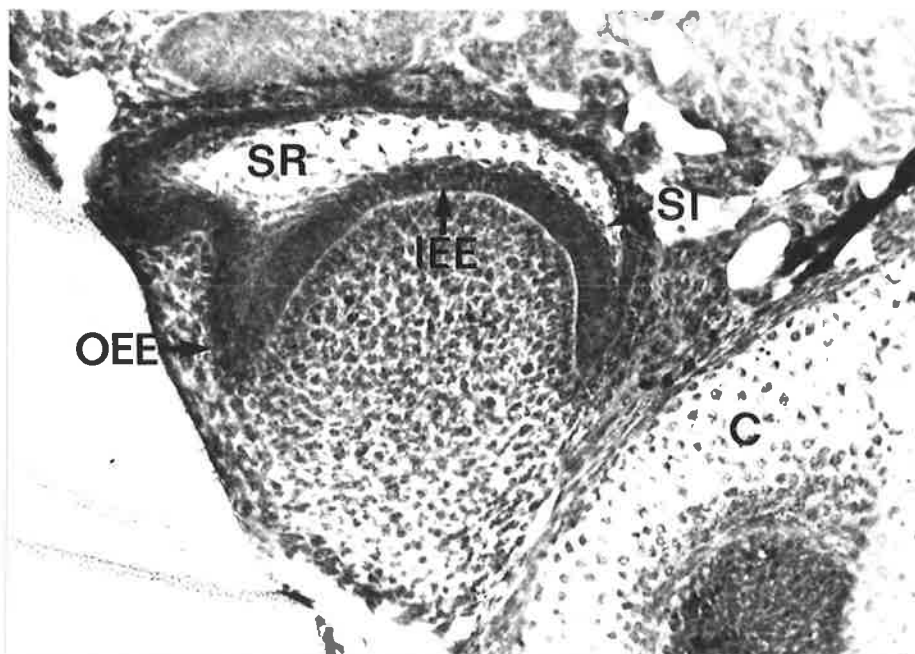
Fig.60 13½ day old explant, cultured for 7 days.
Tooth germ in early bell stage with inner and outer enamel
epithelia(IEE, OEE), stellate reticulum(SR) and stratum
intermedium(SI). Cartilage(C) could also be seen nearby.
(Haematoxylin & Eosin, x200)
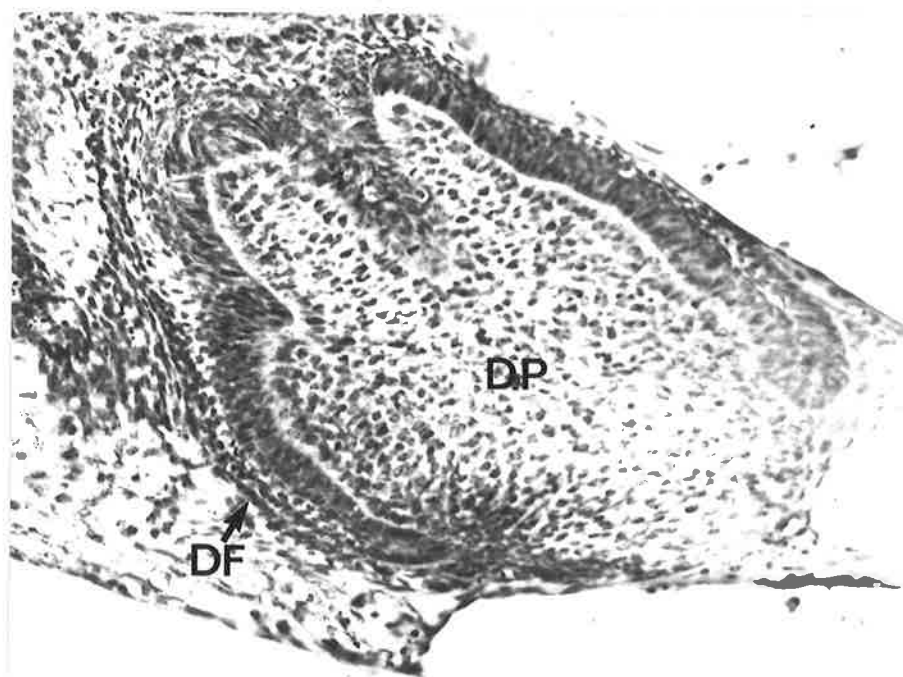
Fig.61 13½ day old explant, cultured for 10 days.
Tooth germ in late bell stage.
Dental papilla(DP), Dental follicle(DF).
(Haematoxylin & Eosin, x200)

at various levels. The outline of the future dento-enamel junction was
demarcated by a cell-free zone. Underneath this, the pre-odontoblasts
were seen arranged as a layer of cells on the inner aspect of the
enamel organ, while cells within the centre of the dental papilla appeared
loosely arranged. The future dental follicle was present as an ambient
structure, 2-3 cells thick and continuous around the base of the tooth
germ at the cervical loop area. At this stage of development, no secretory
activity was detectable although no doubt the cells were actively engaged
in the preparatory stages of cytodifferentiation.

After 15 days *in vitro* (Fig. 62)

Explants were cultivated in both sorts of media but greater success
was achieved with the BGJb medium. The molar tooth germ, in transverse
section, had begun hard tissue matrix secretion, mainly predentine. No
enamel matrix was yet detectable although the telling signs of ameloblast
differentiation, such as polarization of the nuclei were evident. The
rest of the explant was made up by epithelium undergoing squamous meta-
plasia and keratinization.

After 16 days *in vitro* (Figs. 63, 64)

Explants in this, and subsequent groups, were grown solely in
BGJb medium. Both the explants, on recovery, were remarkably healthy
when compared to other groups. The tooth germ, in transverse section,
was unmistakably in the advanced stages of hard tissue differentiation.
At the region of future cusp formation, the ameloblasts have assumed
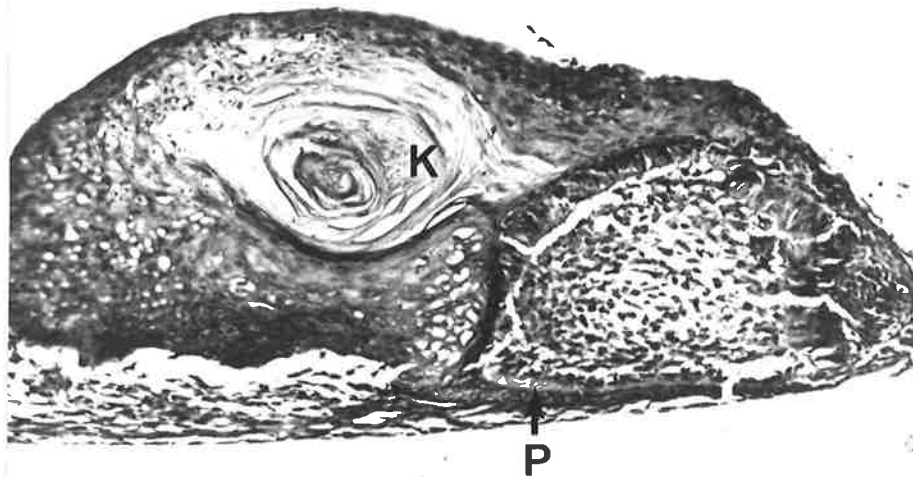
Fig.62 13½ day old explant, cultured for 15 days.
Tooth germ has begun to secrete predentine(P).
Keratin(K). (Haematoxylin & Eosin, x160)
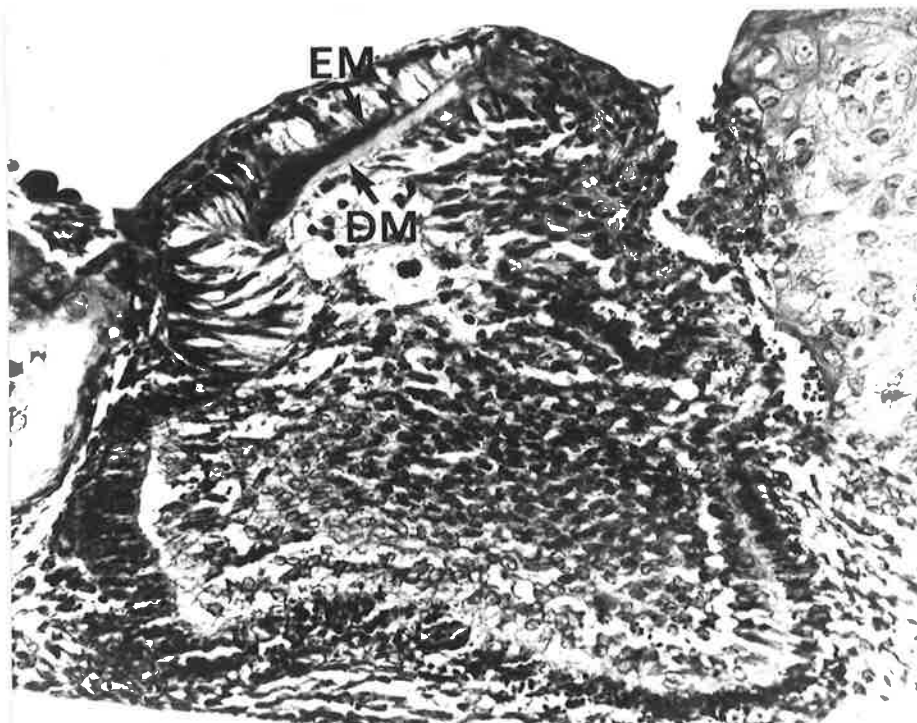
Fig.63 13½ day old explant, cultured for 16 days.
Tooth germ in advanced stage of differentiation. Enamel
matrix(EM) laid on top of a layer of dentine matrix(DM)
(Haematoxylin & Eosin x300)
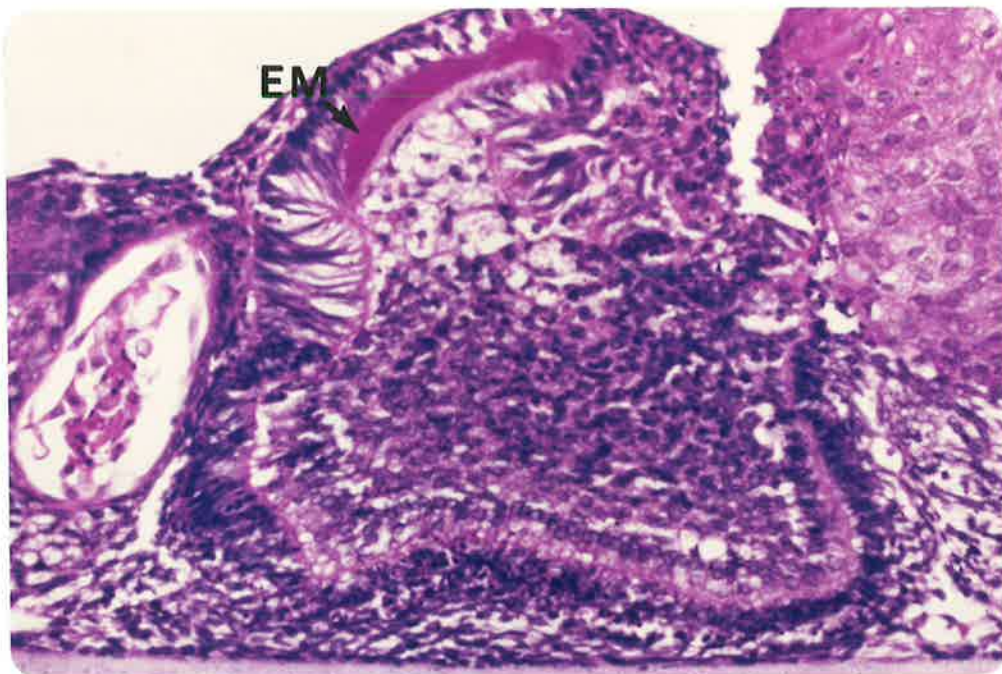
Fig.64 13½ day old explant, cultured for 16 days.
More advanced hard tissue matrix secretion, the enamel matrix
(EM) is possibly mineralized.
(Haematoxylin & Eosin, x300)
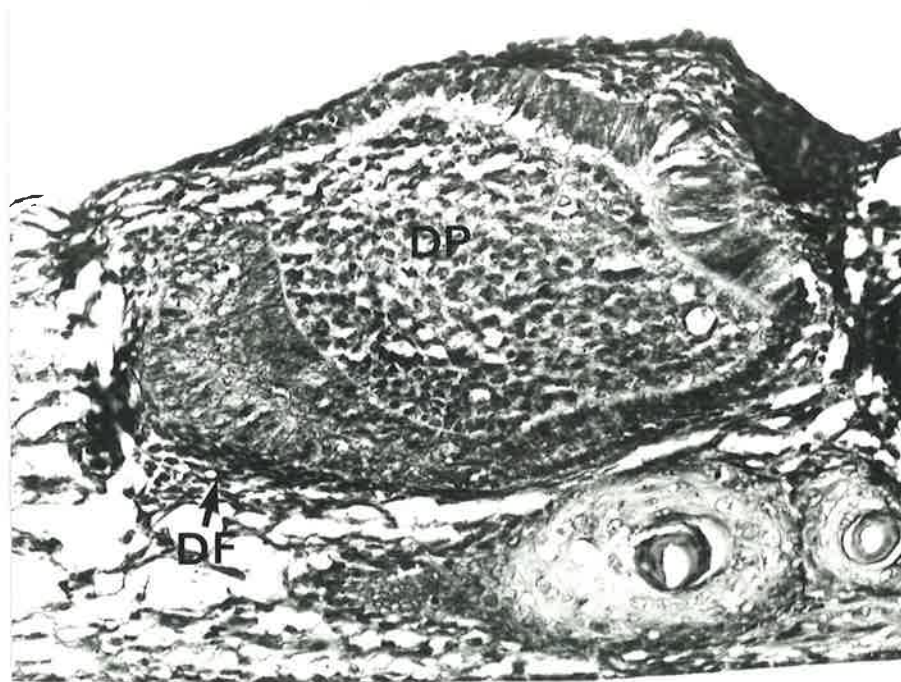
Fig.65 13½ day old explant, cultured for 17 days.
Late bell stage of tooth development, no hard tissue differen-
tiation. Dental follicle(DF), Dental papilla(DP).
(Haematoxylin & Eosin, x200)

a tall columnar appearance, with polarization of the nuclei toward the apical ends. Numerous cellular processes could be seen branching into the layer of secreted enamel which was no doubt undergoing mineralization (Fig. 63). Apposite to the enamel was a layer of predentine, secreted by the retreating odontoblasts. Underneath this was a diffused stroma of pale staining matrix, and trapped within were a few odontoblasts.

Elsewhere, the differentiated ameloblasts and odontoblasts were separated by a thin layer of hard tissue matrix, from which the secreting cells appeared to be retreating.

At other areas, within the same tooth germ, there was advanced hard tissue formation(Fig. 64), and whereas predentine could be present unaccompanied by enamel(Fig. 63), the reverse situation was never presented. The cells of the dental papilla were cuboidal and numerous mitotic figures could be seen within their nuclei. The remaining parts of the explant were mainly filled with cartilage and keratinizing epithelia.

After 17 to 20 days *in vitro*(Figs. 65, 66, 67, 68)

Explants grown for 17, 18, 19 and 20 days all exhibited tooth germs on recovery. However, when compared with explants cultivated for 16 days(Figs. 63, 64), they were retarded and histodifferentiation of the hard tissues did not reach up to equivalent levels. The tooth germs were all relatively healthy, but for some reason or other, enamel matrix secretion was suppressed even though they were all cultured for periods longer than 16 days. The ameloblasts were tall columnar and exhibited polarization of their nuclei, but very little enamel matrix, if any, was
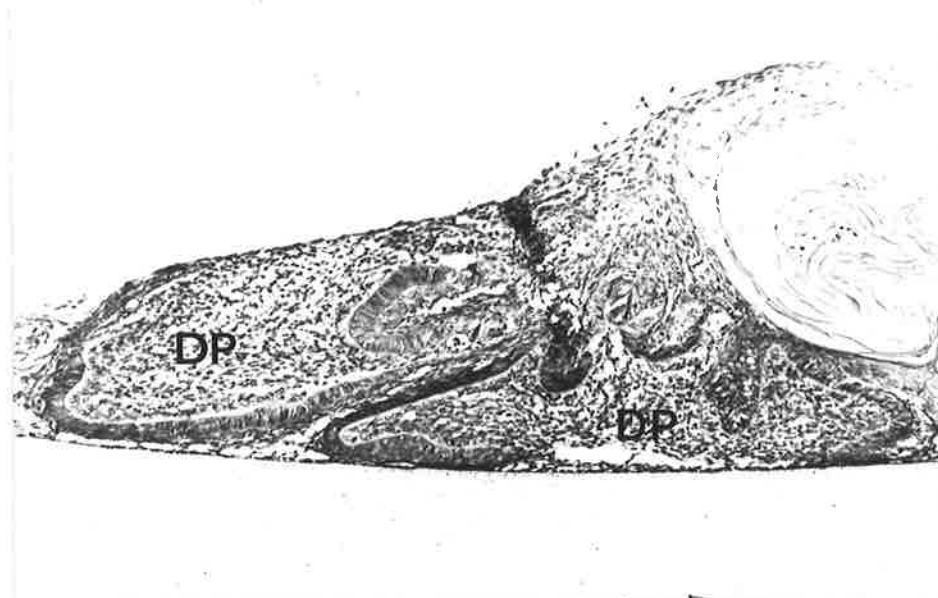
Fig.66 13½ day old explant, cultured for 18 days.
Two molariform tooth germs could be seen adjacent to one
another. No hard tissue formation.
Dental papilla(DP). (Haematoxylin & Eosin, 125)
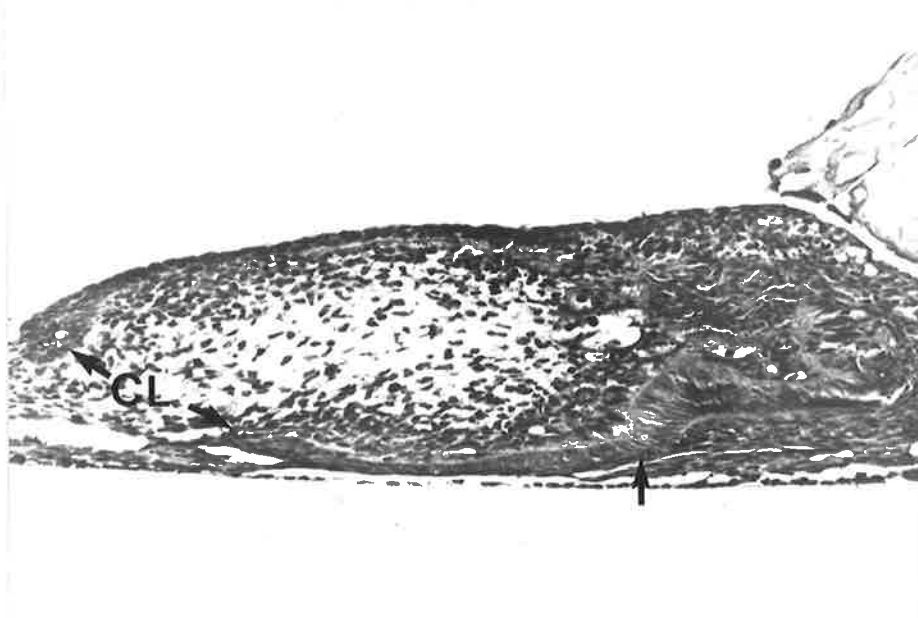
Fig.67 13½ day old explant, cultured for 19 days.
Ameloblast differentiation(arrow) has begun but no enamel
matrix secretion yet. Cervical loops(CL).
(Haematoxylin & Eosin, x200)
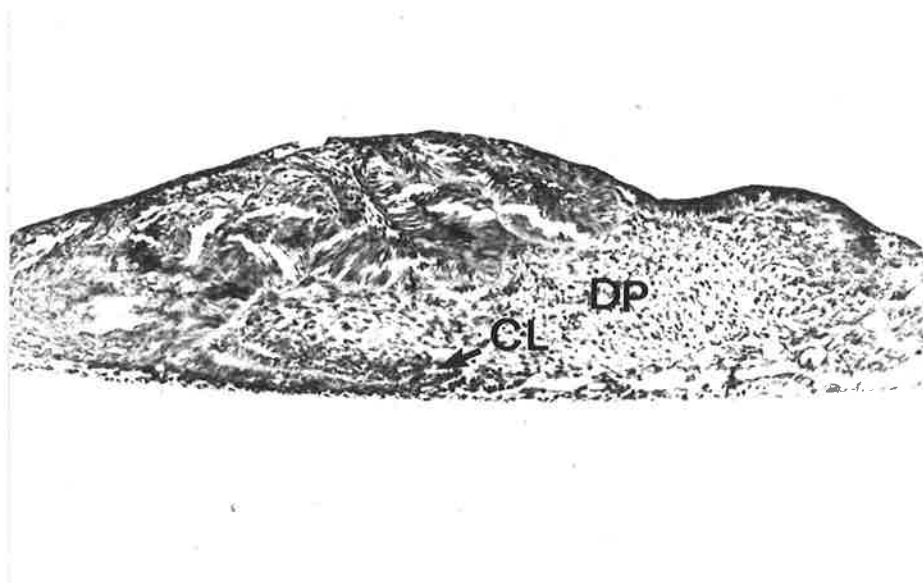
Fig.68 13½ day old explant, cultured for 20 days.
Molariform tooth germ but no hard tissue matrix secretion.
Dental papilla(DP), Cervical loop(CL).
(Haematoxylin & Eosin, x100)
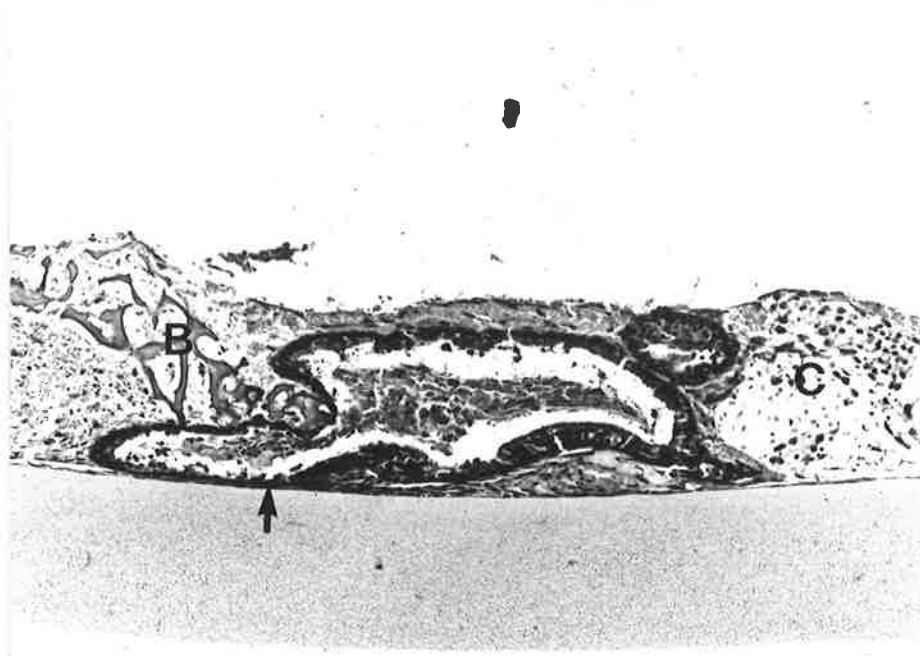
Fig.69 13½ day old explant, cultured for 30 days.
No tooth germ formation, instead bone(B), cartilage(C)
and epithelial cyst(arrow) were present.
(Haematoxylin & Eosin, x125)

detectable at their brush borders.

Predentine however, could be seen as a thin layer of palely stained matrix next to the odontoblast layer in all the explants. The odontoblasts were similarly differentiated and the remainder of the dental papilla was consist of loosely organized cuboidal cells.

The dental follicle was, on most occasions, detectable as an investing layer of cells around the tooth germ(Figs. 65, 67). As for crown morphology, the tooth germs were mostly molariform(Figs. 66, 68) but in other cases (Fig. 65, 67), this was less evident due to the aberrant plane of sectioning. Occasionally, more than one tooth germ was present within a single explant(Fig. 66)

After 30 days *in vitro*(Fig. 69)

In this solitary case, the explant failed to produce teeth but instead, a cystic structure lined by epithelium was produced. Within its cavity, a conglomerate of amorphous material and non-specific cells were present. Bone of the cancellous type and cartilage were both seen but no dental tissues were present.

Chapter 6. DISCUSSION

NEURAL CREST CELLS - THE BURDEN OF PROOF

Ideally, the NC cells ought to be marked with a radioactive label and their subsequent movement into the jaws traced by autoradiography. Due to reasons mentioned earlier, such an approach is fraught with numerous technical difficulties, the lack of a permanent cell marker being one. In the absence of a direct manipulation of NC tissue in this study, any claims attached to a population of cells found wandering around the jaws is open to criticism. However, the NC can be proved guilty by association and such an approach will be more appropriate for the present study.

THE SIGNIFICANCE OF GLYCOGEN CONTENT

From the outset, glycogen content was singled out as the main criterion for the specific identification of NC cells in the tooth bearing areas(see Introduction and Review of Literature). On that account, this study has been remarkable successful; although previously, the relationship between glycogen-content and NC cells has been established only at the light microscope level(MILAIRE, 1959; POURTOIS, 1961; TEN CATE, 1969).

In the present study, the ultrastructural identification of glycogen within the cell was not a problem, being based on morphological criteria previously spelled out by other workers using liver tissue and pure glycogen pellets(REVEL, NAPOLITANO & FAWCETT, 1960; REVEL, 1964; DE BRUIJN, 1973; DE BRUIJN & DEN BREEJEN, 1975, 1976). The feeble demonstration of

glycogen in tissues fixed with tannic acid-glutaraldehyde can be
attributed to the decreased pH; maximum glycogen preservation is
attained under alkaline conditions(HAYAT, 1975). This being considered,
the ultrastructural demonstration of glycogen, especially in tissues
fixed with glutaraldehyde alone, has been signally unambiguous and
reliable - being present in 13½ day old mesenchyme throughout but absent
in 12½ day old tissues.

Such a dramatic change, occurring within 24 hours, in the
cytological character of dental mesenchyme can only be brought about
by a migratory process, or cellular differentiation; or both. On the
strength of other features evinced by these cells, there is more
evidence to support the migration theory. Glycogen is a common feature
of migrating cells, as a ready source of potential energy. It has been
found in proliferating outer enamel epithelium(TROTT, 1965); prickle
cell layer of regenerating epithelium(TROTT & PEIKOFF, 1963) and in
cells which become fibrogenetic under certain experimental conditions
(ROSS & LILLYWHITE, 1965). More recently, glycogen content has been described
as a distinctive feature of mesenchymal cells actively involved in the
reorientation of palatal shelf rotation(INNES, 1978). In all the above
events, glycogen is believed to fulfill an energy-supplying role in
cellular locomotion. Such an interpretation is compatible with data drawn
from the present study.

The concentration of glycogen was highest within mesenchyme found
at some distance away from the dental lamina(in 13½ days); the cells in
these regions were separated by large intercellular spaces occupied by
a rich extracellular matrix(ECM). As the mesenchyme approached closer
to the thickened epithelium, there was an abrupt drop in glycogen content,
simultaneous with a decrease in ECM concentration; the cells in this area

have clearly condensed together to form a closely packed mass. Thus,
at the conclusion of migration, the reduction in glycogen content
could be due to progressive utilization as the cells tail off their
migratory activity.

Such a theory is not contradictory in the face of concepts currently
believed to govern NC cell migration. In all probability, intracellular
glycogen is a product of cellular maturity as the NC cells verge closer
to their migratory destinations; NC cells at the start of migration are
recognized to be empty of any cytoplasmic characteristics - at least in
the chick(BANCROFT & BELLAIRS, 1976). A further clue is provided in the
work of LE DOUARIN & TEILLET(1974) - they found a PAS-positive mucopoly-
saccharide in association with NC cells migrating within the walls of
the gut.

Another possible function of glycogen is that of embryonic induction,
a hypothesis first raised by GLOCK(1940). Previously, glycogen has been
successfully used to induce neural tube formation from the ectoderm of
amphibian gastrula(WADDINGTON, NEEDHAM, NOWINSKI & LEMBERG, 1935). This
has led to the suggestion that glycogen carries within its molecules an
inducer substance capable of evocating tooth development(GLOCK, 1940).
Interestingly enough, glycogen has been found as an extracellular product
of preodontoblast secretion, among the matrix believed to induce
amelogenesis in cervical loop dental epithelia(SLAVKIN, BRINGAS, LEBARON,
CAMERON & BAVETTA, 1969b). In the context of the present study, no glycogen
was found in the extracellular environment, but this does not rule out
the possibility of an inductive role via a secreted matrix molecule.

EVIDENCE FOR CELL MIGRATION

Throughout the review of the literature, a rigorous attempt was made to highlight other features that were commonly found in association with NC cells, albeit non-mammalian ones. Some of these will now be recounted and compared with the ultrastructural observations made in the present study in the effort to construct circumstantial evidence.

The possession of locomotory appendages is an obvious one to begin with. In the present study, the glycogen-rich cells were frequently possessed of filopodia(cellular extensions) extending for long distances adjacent to one another and at the sites of contact, produce electron-dense cell junctions or plaques. At other instances, the terminal ends of the filopodia were found abutting on neighbouring cell bodies. However, no ruffled membranes(an *in vitro* artefact, BARD & HAY, 1975) were seen at their free ends. Within the cytoplasm of the filopodia, microfilament bundles and microtubules were frequently observed and in some instances, the microfilaments appear to insert into the electron-dense junctions.

The classical theory of cell locomotion(in fibroblasts) is explained by the contraction of microfilament bundles(WESSELLS, et.al, 1971); this force is in turn exerted on the cell surface causing the overall contraction of the filopodia(a step in locomotion). Alternatively, the contraction of the microfilaments(identified as actin, BURTON & KIRKLAND, 1972) could be translated into cell body locomotion by means of adhesion to a substrate (such as neighbouring cells) forming electron-dense plaques(ABERCROMBIE, HEAYSMAN & PEGRUM, 1971; HEAYSMAN & PEGRUM, 1973). The cells therefore, move in a jerky manner along a substrate by breaking loose adhesions towards the rear and establishing new contacts with the substrate at the

leading edge(ABERCROMBIE, 1961). These events occur in a rapid fashion, mediated by microfilament bundles that assemble(and perhaps dissolve) in a matter of seconds(ABERCROMBIE, 1978) upon the receipt of a signal from cell-to-cell contact(GOLDMAN, 1975).

The formation of new adhesions in front of existing cells can only by brought about by forward protrusion of new filopodia. For this, a forward flow of cytoplasm is required and microtubules are thought to provide the necessary channels for directing the flow(ABERCROMBIE, DUNN & HEATH, 1977; FREED & LEBOWITZ, 1970).

So far, a migratory role has been ascribed to the glycogen-rich cells on the bases of their ultrastructural features and the possession of locomotory appendages. However, it should be pointed out that the precise mechanisms by which NC cells propel themselves are yet unknown. Presently, only a handful of direct studies(TEM) on NC cells are available; even then, they concern NC cells during the initial stages of migration in non-mammalian embryos. Nonetheless, microfilaments and microtubules have been found within the initially migrating NC cells(BANCROFT & BELLAIRS, 1976; MORAN et. al, 1978; LOFBERG & AHLFORS, 1978) although in sparse quantities only. Similarly, the presence of electron-dense plaques and specialized contacts between adjacent NC cells have been widely reported(EBENDAL,1977; LOFBERG & AHLFORS, 1978); and cell processes have been described under a variety of terms viz. filopodia, lamellopodia, microspikes, ruffled membranes. The extensive changes in cellular morphology(bipolar to stellate) has led to the conclusion that in early NC migration, cellular motility is brought about by changes in the external environment and responded to by alterations in cell shape; rather than by contraction in the cytoskeleton(BANCROFT & BELLAIRS, 1976).

One of the more interesting observations in the present study was the regional differences in ECM content within mesenchymal regions of 13½ day old dental anlagen. In the proximal areas, close to the dental epithelium and corresponding to the cessation of cellular migration, there was a distinctive fall in ECM concentration. Further distal, the ECM was more intensely stained, appearing as thread-like material with electron-dense blobs, in between widely separated cells. In the absence of a histochemical analysis, the exact nature of the ECM material cannot be determined; but tannic acid-glutaraldehyde has been shown to be a GAG precipitant(SOLURSH, FISHER & SINGLEY, 1979; SLAVKIN & BRINGAS, 1976). According to this interpretation, the thread-like material(2 nm diameter and 5 nm in length) seen in this study are probably hyaluronic acid while the electron-dense blobs are proteoglycan aggregates.

The above is a significant finding because the association of hyaluronic acid(and other GAG) with NC cell migration has been a widely reported phenomenon(see Review of the Literature). In the context of the present study, hyaluronic acid concentration was greatest in the areas active in cellular migration and noticeably rich in glycogen. In areas of cellular condensation(arrest of cell migration), the ECM content was minimal except for the region immediately underneath the basal lamina. Hyaluronic acid has been suggested to play an important role in cell migration(TOOLE, 1972), postponement of cellular differentiation(TOOLE, 1973), the maintenance of cell-free spaces(PRATT et.al, 1975) and the mediation of tissue interactions. Any one, or all of the above, could be the function of the rich ECM found adjacent to the glycogen-rich cells in this study.

Apart from the ECM and cell-to-cell contacts, the possible role of blood capillaries as a substrata for migration is worthy of consideration.

Such an observation was first made by NODEN(1975) who noted the use of
blood vessesl and nerve fibres as substrata for the migration of chick
NC cells into the maxillary processes. This would explain   the
occasional finding of glycogen-rich cells around the blood capillaries
of 12½ day old mesenchyme. This theme will be enlarged in a latter part
of the discussion.

The presence of microvesicular sacs beneath the cell membrane is
a more difficult finding to explain. These structures were found in
cells of both 12½ and 13½ day old tissues, and a definitive role cannot
at present be ascribed to them. In fact, the occurrence of similar
structures, termed aggregated vesicles, has been noted in dental
mesenchyme of mouse embryos from 10 days post-conception onwards(MEYER,
FABRE, STAUBLI & RUCH, 1977). These workers have suggested that the
vesicles are the bearers of inductive stimuli, a view that is to be
disagreed with in the absence of concrete evidence. Similar structures
beneath the cell membrane have been reported in a variety of tissues:
as 'growth cones' in growing nerve tips(DEL CERRO & SNIDER, 1968), as
'adhesive organelles' in glial cells(SPOONER, YAMADA & WESSELLS, 1971)
and in ovarian follicles(ESPEY, 1971). Such a widespread occurrence of
this structure in disparate tissues would caution against ascribing a
single function to it, although its function as a locomotory organelle
in extending nerve fibres is a near certainty(BRAY, 1973; BRAY & BUNGE,
1973).

In sum, the designs of the present study has not permitted a
conclusive identification of NC cells but circumstantial evidence is
sufficiently weighty to suggest that the glycogen-rich cells are of
NC origin. If such were the case, then it would not only confirm the
putative contribution of NC cells to mammalian odontogenesis; but would
also raise a number of interesting questions, dealt in the latter parts
of the discussion.

ORGAN CULTURE

TIMING OF NEURAL CREST CELL ARRIVAL

A crucial issue in the interpretation of data from the present

study is the exact timing of NC cell arrival at the tooth bearing

regions. Assuming that the glycogen-rich cells originate from the NC,

then one is forced to conclude that in C3H mice, the NC cells arrive

at the maxillary molar regions sometime between $12\frac{1}{2}$ and $13\frac{1}{2}$ days

postconception. Such a conclusion is further borne out by the observa-

tion that, in my hands, none of the explants taken from $12\frac{1}{2}$ day old

embryos developed teeth in culture. On the other hand, a high percentage

(75%) of explants removed from $13\frac{1}{2}$ day old embryos produced molariform

tooth germs. The association between the ability to form teeth and the

presence of glycogen-rich cells in the explants is highly suggestive of

an indispensable role, assumed by the glycogen-rich cells, whatever their

origin might be. The inability of the remaining 25% to form teeth is not

due to lack of a contribution by glycogen-rich cells(since they were

shown to be present in the zero-time controls), but more likely due to

tissue trauma and/or subversive culture conditions.

Other investigators have suggested a much earlier developmental

time-table as regards NC arrival into the tooth bearing areas. Almost

without exception, the inferences were based on observations of tooth

formation or lack of it in extirpated dental anlagen grown in culture.

Predictably, a wide range of dates have been quoted by different

investigators. In the mouse mandible, RUCH, KARCHER-DJURICIC & GERBER

(1973) suggested that NC cells migrate into the tooth bearing areas at

the 9th day of gestation, this conclusion was derived at from culture

studies of extirpated mandibular arches. POURTOIS(1964) quoted 8½ days
as the day of NC arrival but he was unable to cultivate teeth from
explants until the 12th day of gestation. Both DRYBURGH(1967) and
MILLER(1969) were able to grow teeth from 9 day old mouse mandibles
but HAY(1961) was unsuccessful in culturing mandibular incisors and
molars even when the explants were 13 days old and exhibited thickening
of the oral epithelium. Greater success was achieved with explants
older than 13 days and that exhibited a definite formation of the dental
lamina(HAY, 1961). However, GLASSTONE(1963) was able to cultivate teeth
from 11 - 12 day old mouse mandibular tissues, and by this stage the
shape of the tooth was found to be fully determined.

The disparate results given above are attributable to various factors.
The chronological age of the embryo is an unreliable measure of develop-
mental status and should not be the sole staging criteria. GLASSTONE(1963)
has pointed out that different litters of the same chronological age
may have differing developmental status; indeed within the same litter,
some embryos might be precocious while others retarded in development.
There are also obvious differences in the developmental timetable between
different strain of mice(SLAVKIN, 1974). Finally, one should always bear
in mind that on the whole, development of teeth in the mandible is always
in advance of the maxilla, engendering an earlier migratory event in the
mandible. Given the lag in maxillary tooth development, it seems probable
that NC cell migration into the maxillary molar bearing areas occurs
sometime after 12½ days. Even then, if an explant was large enough, it
it possible to include NC cells just entering into the maxillary process
but not quite at the presumptive dental regions yet. This would account
for the greater incidence of tooth formation when large explants, such
as whole mandibles were cultivated(POURTOIS, 1964; DRYBURGH, 1967; RUCH

et. al, 1973).

It is unfortunate that the above mentioned studies have not quoted the developmental status of the explants(at zero time) in histological terms, otherwise a profitable comparison with the results of the present study could be made. From the data of the present study alone, the exact timing of NC cell arrival into the tooth bearing areas(maxillary molars) could be placed as sometime in between 12½ and 13½ days in gestation. To an extent, this is corroborated by the observation that NC cells (destined for pigment formation) do not penetrate the ectoderm of black mice until the 12th day of gestation(MAYER, 1973).

However, it is possible that an earlier NC cell population, destined to form cartilage and connective tissue, has migrated into the mesenchymal areas of the jaws before day 12½. Tooth formation, being a late occurring event – by comparison to osteogenesis and chondrogenesis –is then furnished by a secondary population of NC cells, rich in glycogen, after day 12½. Such an interpretation is by far the more eclectic one; and would account for the presence of cartilage and bone in 12½ day mesenchyme grown in culture.

HARD TISSUE FORMATION *IN VITRO*

Tooth germs have been cultivated *in vitro* for quite some time now (GLASSTONE, 1936) and numerous organ culture techniques have since been successfully applied(SZABO, 1954; HAY, 1961, GLASSTONE, 1965). Although tooth germs can be kept viable for long periods of time(up to 54 days, NIIZIMA & CATTONI, 1958), complete differentiation of the hard tissues was seldom seen until recently(KOCH, 1967; WIGGLESWORTH, 1968; THESLEFF, 1976).

In the older culture techniques, enamel matrix was seldom secreted and even if it did, it would fail to calcify(GLASSTONE, 1938; 1964; HAY, 1961). The blame was often laid on the culture system, whether it be the medium or gaseous atmosphere. A variety of improvements have since been suggested to ensure constant enamel formation in organ culture; among them a high content of ascorbic acid, embryonic extract, serum; and a high oxygen tension(WIGGLESWORTH, 1968; KOCH, 1972; THESLEFF, 1976). Others, on the other hand, have reported the detriment of having serum, antibiotics, carbon dioxide and embryonic extracts in the culture set up and have advocated for their removal before any dentine or enamel matrix secretion can be expected(YAMADA, BRINGAS, GRODIN, MACDOUGALL, CUMMINGS, GRIMMETT, WELIKY & SLAVKIN, 1980).

The exact requirements for full enamel maturation remain unknown, but among chemically defined media, BGJb medium(BIGGERS et. al, 1961) in particular has proved itself especially when supplemented with ascorbic acid and animal serum(WIGGLESWORTH, 1968, THESLEFF, 1976). Under the above conditions, the secretion of enamel matrix was a repeatable observation and often comparable to tooth germs grown as

transplants, for example in the abdominal wall(HUGGINS, McCARROLL & DAHLBERG, 1934), in the anterior chamber of the eye(FLEMING, 1952; KOLLAR & BAIRD, 1970a,b) and in the chick chorio-allantoic membrane (SLAVKIN & BAVETTA, 1968). The present study has once again confirmed the suitability of BGJb medium in supporting enamel matrix secretion, although it was not a repeatable finding. When Medium 199 was used, no hard tissue secretion was observable.

In the past, the main part of the attention has been focussed on the culture conditions, and not on the age of the explants initially grown. In the above mentioned studies, the successful ones have invariably employed tooth germs in the late bell stage of development. WIGGLESWORTH (1968) used 17 day old tooth germs from rat embryos; obtaining enamel secretion after 12 days in culture. THESLEFF(1976) removed tooth germs from 17 day old mouse embryos and found enamel matrix secretion as early as the 7th day in culture(normally, enamel secretion begins on the 20th day of *in vivo* development). YAMADA et. al(1980) used a different medium (Eagle's Minimum Essential Medium) and obtained enamel and dentine matrix secretion after culturing 17 day old mouse tooth germs for 10 days. However, due to the omission of serum from the medium, no calcification was seen.

In the present study, the explants were hardly past the dental lamina stage(13½ day old embryos), and yet a calcified enamel matrix was seen after 16 days in culture. Admittedly, only one specimen out of nine successful explants in Study 2(BGJb medium) showed this condition, thus; the finding must be a fortuitous one. Nevertheless, it does not detract from the fact that dental rudiments extirpated at as early as the tooth bud stage(13½ days) are, in the appropriate culture conditions, capable of differentiating fully into calcified enamel and dentine.

The exact conditions surrounding this occurrence are not known, and further experiments with strict controls will have to be instituted before the factors responsible for hard tissue matrix formation(and mineralization) can be tracked down. It is highly probable that the increased serum content(20%) in conjunction with the use of BGJb medium has encouraged ameloblast differentiation, a point stressed by THESLEFF (1976). However, there is no support for the contention that the secretion enamel matrix is conditional upon the presence of embryo extract.

The role of ascorbic acid as a collagen-enhancer is universally agreed but the precise concentration is unknown. In the present study, the medium used was equipped with 50 mg/L, a small quantity when compared with other studies. The antibiotic Kanamycin was added to the medium, but only sparingly(50 µg/ml). As for pH, temperature, buffer systems, number of explants per given volume of medium; this study has not produced any findings to change the current opinion(YAMADA et. al, 1980).

EPITHELIAL PROLIFERATION AND KERATINIZATION

A constant feature of the explants, grown in culture, was the extensive degree of epithelial proliferation and random invasion into the underlying mesenchyme. This was more commonly observed in explants taken from 12½ day old embryos, where the unanimous absence of tooth formation was matched only by the consistent presence of keratinizing pearls and cysts. The behaviour of 12½ day old oral epithelia(*sans* glycogen-rich cells) was strongly reminiscent of similar findings reported by KOLLAR & BAIRD(1970b). In their study, 12½ day old oral epithelium(removed from its normal inductive environment) was grown alongside foot pad mesenchyme and the resulting tissue displayed no evidence of tooth formation, only kertinizing cysts and randomly proliferating epithelia.

There was one other finding, in the present study, that would merit discussion. When 12½ day old epithelia was grown in culture for 10 days, the epithelial proliferation resembled that of a molar enamel organ; even though no ameloblasts or odontoblasts were seen. A similar finding has been reported by KOLLAR & BAIRD(1970b), who on recombining molar epithelium with foot-pad mesenchyme, obtained an epithelial proliferation in the form of an enamel organ complete with stellate reticulum, but no amelogenesis was observed. These observations would suggest that the information for the determination of crown form are resident within dental epithelium at an early age, and even if the epithelium was robbed of its accompanying mesenchyme, it was independently capable of carrying out its morphogenetic designs, but in a limited way only.

EPITHELIAL-MESENCHYMAL INTERACTIONS IN ODONTOGENESIS: A CRITICAL APPRAISAL

A survey of the literature has revealed that there is no single unifying theory that will explain all the observable events of odontogenesis in a convincing manner. This is commonplace in all areas of scientific enquiry, especially when dealing with a structure as complex as the developing tooth. However, a certain measure of blame must be ascribed to dental histologists who have in the past, failed to separate fact from postulate. It was not uncommon to come across reports that successively incorporate a previous hypothesis into a fact by quoting it repeatedly.

A topic such as embryonic induction is an elusive one because available tools of experimentation are only capable of depicting a dynamic event in a static fashion. This is especially true in the study of tissue interactions by *in vitro* culture of tooth rudiments; relying on the assumption that it is possible to recognise the end product of an interaction as a morphological or biochemical entity. This is not necessarily correct during the earlier aspects of tooth formation, when interactions result in non-tangible developmental changes, such as restriction of potential and acquisition of competence. Therefore, in the absence of rigorous experimental testing, one is entitled to view current theories of epithelial-mesenchymal interactions in odontogenesis with suspicion.

The discovery that epithelial-mesenchymal interactions play a large and necessary part in the initiation and maintenance of various organ systems(e.g. salivary gland, kidney tubules, thyroid, hair follicle) led many to postulate equivalent events in tooth formation. Existing evidence

has vindicated some of these theories but others unfortunately remain unsubstantiated.

Right from the appearance of the dental epithelium to the secretion of enamel matrix, no less than four inductive events are presumed to have transpired(KOCH, 1972; SLAVKIN, 1974; RUCH & KARCHER-DJURICIC, 1975). Allowing for minor variations, these events can be succinctly stated in the following manner:

1) NC ectomesenchyme act on oral epithelium to induce epithelial thickening.

2) Inner enamel epithelium induces the dental papilla to differentiate into odontoblasts.

3) Odontoblasts induce cytodifferentiation of ameloblasts from the inner enamel epithelium.

4) Predentine induces enamel matrix secretion by ameloblasts.

Diagrammatically, the chain of events can be represented as:
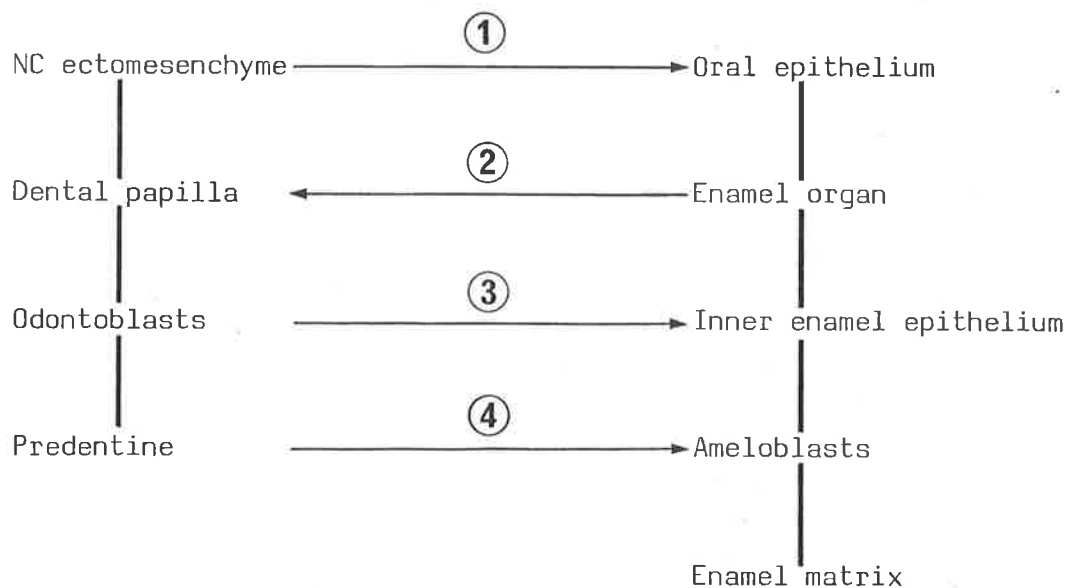
Fig. 70

*The horizontal arrows indicate the direction of the inductive event while the vertical lines delineate the progression in histodifferentiation.

Apart from an isolated instance(RUCH et. al, 1973), little attempt
has been made to provide experimental evidence for Step 1. Since new
information from the present study has contributed to a renewed under-
standing of this phase of tooth development, it will be left to the
last for a lengthier discussion.

It has been suggested(RUCH & KARCHER-DJURICIC, 1971, 1975; RUCH,
KARCHER-DJURICIC & THIEBOLD, 1976) that the inner enamel epithelium
plays a special role in the initiation and maintenance of odontoblast
differentiation in Step 2, implying a directive sort of interaction.
KOCH(1967) has shown that in the transfilter situation, normal tooth
development occurs only when both epithelium and mesenchyme are juxtaposed
across each other. This has led to the belief that inductive information
is transmitted by the epithelium to elicit differentiation of mesenchyme
into odontoblasts. Since the basal lamina *in situ* is normally intact,
heterotypic cell-to-cell contact between epithelium and mesenchyme could
not have occurred; and so the ECM found in between has been implicated
as the most likely bearer of inductive stimuli(SLAVKIN, LEBARON, CAMERON,
BRINGAS & BAVETTA, 1969a). This matrix, consisting of collagenous material
and membrane-bound dense bodies, was shown capable of aligning cell
suspensions of either incisor epithelium or mesenchyme into a secretory
arrangement. However, matrix secretion was not seen and undue importance
should not be attached to the ECM(found adjacent to preodontoblasts)on
the basis of the above finding alone. In fact, under similar experimental
conditions, THESLEFF(1978) has also evoked a similar arrangement from
dermal fibroblasts.

Since NC ectomesenchyme are thought to arrive into the tooth
forming regions fully determined to become odontoblasts, a permissive

environment is all that might be required. Unequivocal evidence has now
been provided that this permissive interaction is provided for by the
basal lamina located beneath the inner enamel epithelium(THESLEFF, 1977;
THESLEFF, LEHTONEN, WARTIOVAARA & SAXEN, 1977). Transfilter experiments
have also shown that a collagen matrix alone will not do, but a direct
contact of the preodontoblast cell process with an intact basal lamina
in its three-dimensional form is requisite before odontoblast differen-
tion(THESLEFF, 1978; THESLEFF, LEHTONEN & SAXEN, 1978).The basal lamina
is in turn dependent upon the adjacent mesenchyme; recent evidence has
shown that the formation of a new layer of basal lamina by epithelia
in vitro does not proceed unless and until it is juxtaposed against
dental mesenchyme(THESLEFF et.al, 1978; KARCHER-DJURICIC, 1979).
Incidentally, the disruption of the basal lamina architecture by Vitamin A
or diazo-oxo-norleucine(DON) is known to prevent odontoblast differentia-
tion(HURMERINTA, THESLEFF & SAXEN, 1979, 1980). In conclusion, there is
evidence to support the inductive event(Step 2) depicted in Figure 70,
but the interaction is a permissive one, and not a directive one as the
arrow would suggest.

Evidence for tissue interactions represented by Steps 3 and 4(in
Figure 70)is patchy and conflicting although there can be no doubt that
a directive influence is at work here. Early observations of tooth
development in ectopic sites(HUGGINS et. al, 1934) and in transfilter
experiments(KOCH, 1967) have revealed that ameloblast differentiation
does not occur unless secreting odontoblasts are first present.

KOLLAR & BAIRD(1969, 1970a, b) have shown that the dental mesenchyme
is the specific component as far as Steps 3 & 4 are concerned. By
recombining molar and incisor mesenchyme with epithelia from a variety

of sources(both dental and non-dental), tooth form and shape was always directed by the source of the mesenchyme - whether incisor or molar. The source of the epithelium was non-specific, and even foot-pad epithelium could be induced to differentiate into ameloblasts. These experiments, however, have not revealed the nature of the inductive stimuli although one should be reminded that the procedure of trypsin digestion would have removed the pre-existing basal lamina and the formation of a new basal lamina in the recombinants would have been subject to the organising influences of the dental mesenchyme. In other words, had the epithelial basal lamina been intact before recombination, would the mesenchyme still have as much say in terms of directing the crown form.

Attempts to pinpoint the exact direction of inductive stimulation in the *in vivo* situation have been met with much less success. To begin with, investigators are not agreed on the exact sequence of events leading to enamel matrix secretion and the criteria of ameloblast differentiation vary widely. In addition, reports are often based on experimental data obtained from teeth of dissimilar ages and species.

The extracellular matrix interposed between differentiating odonto-blasts and preameloblasts has been reported to be made up by a motley of inductive constituents. Each constituent has been advocated as a bearer of inductive information; during this stage of development when no heterotypic cell-to-cell contacts are seen. SLAVKIN et.al(1969b) have proposed that the ECM made up by microfibrils, glycoproteins and collagen mediate the transfer of inductive information across the basal lamina, initiating amelogenesis. Subsequent to this, membrane-bound matrix vesicles(containing RNA) and odontoblast cell processes are

believed to be involved in the break down of the basal lamina, making direct cell-to-cell contacts possible(SLAVKIN & BRINGAS, 1976; SLAVKIN et.al, 1977). Taking the hypothesis one step further, SLAVKIN(1978) has proposed that the degradation of the basal lamina serves to trigger off the release of an antigen-like signal which binds to cell membrane receptors in preameloblasts, inducing ameloblast differentiation. This view, if not far-fetched, is unsatisfactory for two reasons. First, descriptive ultrastructural studies are agreed that the breakdown of the basal lamina occurs as a result of penetration by differentiating preameloblasts, not odontoblasts(REITH, 1967; KALLENBACH, 1971; 1976). Second, too much importance may have been placed on the matrix vesicles found in the ECM area next to preodontoblasts(SLAVKIN, et. al, 1972; CROISSANT, et.al, 1975). While they are known to function in the initiation of dentine calcification, the demonstration of RNA within the matrix vesicles has led SLAVKIN and his colleagues to suggest an inductive role, a view challenged by the experiments of GRAINGER & WESSELLS(1974).

The role of heterotypic cell-to-cell contacts as the mechanism of informational transfer has also been discounted by KALLENBACH(1971, 1976). He noted that a basal lamina was often intact even though cytodifferentiation of both preameloblasts and preodontoblasts was well under way. Polarization of the nucleus and formation of vesicles within the preameloblasts were evident at an early stage and in shark, secretion of an enamel-like matrix has been shown to occur in the presence of a perfectly intact basal lamina(KEMP & PARK, 1974; KALLENBACH & PIESCO, 1978). It was conceded however, that the secretion of enamel matrix seen after basal lamina disruption might be dependent upon cell-to-cell contacts between preameloblasts and odontoblasts(KALLENBACH, 1971).

The inductive role of predentine in the stimulation of enamel matrix secretion has been suspected for a long time. Predentine is elaborated by secretory odontoblasts close to the surface of the intact basal lamina. Shortly before calcification of this collagenous matrix, preameloblast processes are known to push through the basal lamina (REITH, 1967). This has led to the suggestion that type 1 collagen, found within predentine, has the ability to polarise ameloblasts(RUCH, FABRE, KARCHER-DJURICIC & STAUBLI, 1974). Suppression of collagen formation with L-azetidine(a proline analogue) was seen to interfere with predentine secretion and consequent loss of ameloblast differentiation.

In sum, the differentiation of ameloblasts appears to be under the directive influences of preodontoblasts and their secretory constituents. Whether this occurs as a two-step(Steps 3 & 4 in Fig. 70) process or more is unknown. This is because the successful characterization of any inductive substance at a molecular level would require testing on embryonic cells and none of the above mentioned tissues have passed this test. In fact, through out the whole range of inductive events in vertebrates, only three substances have so far been identified at the molecular level: the vegetalizing factor acting upon competent amphibian ectoderm(TIEDEMANN, 1973), certain proteoglycans in cartilage formation of somites(KOSHER, LASH & MINOR, 1973) and the mesenchymal factor in pancreatic development (PICTET, FILOSA, PHELPS & RUTTER, 1975).

THE ROLE OF ORAL EPITHELIUM - A NEW POSTULATE

The thickening of the oral epithelium as a consequence of inductive stimulation by NC ectomesenchyme(Step 1, Fig. 70) is an immensely popular belief(see reviews GAUNT & MILES, 1967; SLAVKIN, 1974). This theory is illustrated in Figure 71, taken from a standard text in dental embryology. Direct evidence for such an occurrence in mammalian tooth formation is unavailable, and the successful defence of such a hypothesis would require the demonstration of at least three events; occurring in a sequential manner viz. the arrival of NC cells, transfer of inductive substances and thickening of the oral epithelia.

NC cell migration into the craniofacial regions(of mammalian embryos) has never been demonstrated in a convincing manner, let alone its participation in tooth formation. The studies of POURTOIS(1964) and RUCH et.al (1973) have yielded conflicting results even though mouse mandibular arch was used in both instances. POURTOIS(1964) relied on the histo-chemical demonstration of RNA as a means of identifying NC cell migration which according to him, arrive at the mandibular arch at $8\frac{1}{2}$ days during gestation. However, a visible thickening of the oral epithelium was not evident until the 12th day of gestation and explantation of younger tissues failed to produce teeth. RUCH et. al(1973) thought that the mandibular arch was determined to form teeth from as early as the 9th day of gestation. They induced the formation of a dental lamina by recombining presumptive dental papilla with oral epithelium, but did not define the age of the tissues used in histological terms. This is extremely important because the age of the embryo alone is not a satisfactory measure of embryonic development. Even so, conclusions about NC cell migration can hardly be drawn from studies designed to measure the acquisition of odontogenic competence.
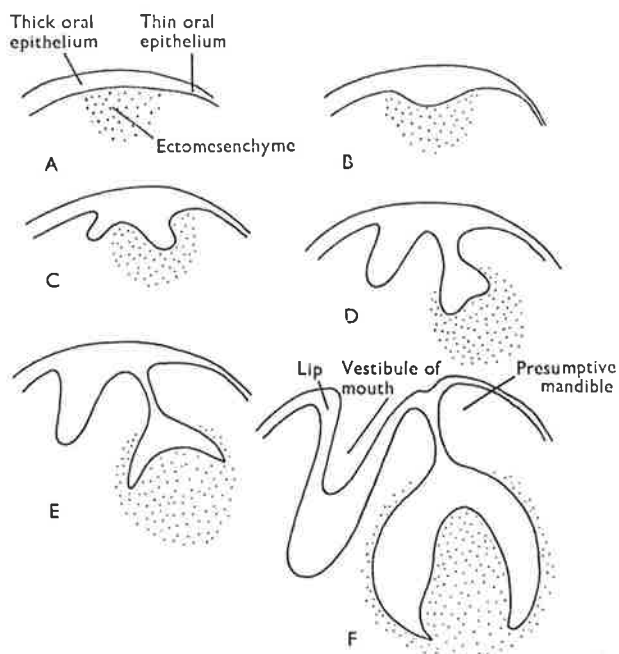
Fig.71 Sequence of events during early tooth formation.
According to this theory, NC ectomesenchyme exerts an
inductive effect, causing proliferation of the oral epithe-
lium(B).
Taken from OSBORN & TENCATE, 1976.
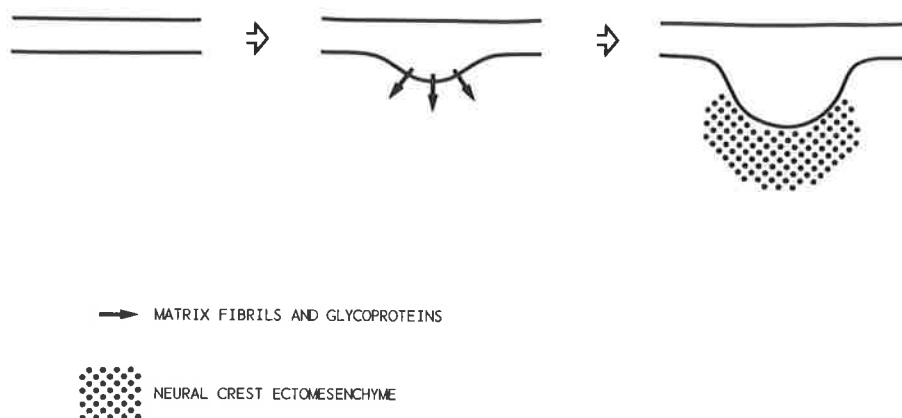
Fig.72 New postulate formulated from the present investigation.
Epithelial proliferation and matrix secretion(aggregation
factor?) is seen to precede NC cell arrival into the tooth
bearing area.

The transfer of inductive stimuli is an extremely elusive pheno-
menon to demonstrate(see previous section); on the other hand, the
thickening of the oral epithelia is readily identifiable. The present
investigation has been successful in the demonstration of NC cell
arrival(in the form of glycogen-rich cells) and the thickening of the
oral epithelia; but not quite in the sequence outlined in Fig. 71(A,B).
Instead, the proliferation of the oral epithelium at the tooth bearing
sites was found to precede, not succeed, the arrival of glycogen-rich
cells by approximately 24 hours. This finding would challenge the sequence
of events popularly depicted in Fig. 71(A,B) and a revised version is
instead   presented in Fig. 72.

As a corollary, the inductive event represented in Step 1(Fig. 70)
should also be regarded as non-existent, since evidence cannot be found
to support it. On the contrary, evidence to refute it has been provided
by KOLLAR & BAIRD(1970b). They found that non-dental epithelium(such
as from the foot-pad) was capable of becoming ameloblasts when grown
adjacent to bell-stage dental papilla. Since foot-pad epithelium would
not have had a previous encounter with NC ectomesenchyme, and yet was
able to differentiate directly into ameloblasts, it would mean that
Step 1 can be safely dispensed with.

If NC ectomesenchyme is not the primary impetus in tooth develop-
ment, then what is? The present study would suggest(in Fig. 72) that
it is the formation of the dental lamina primordium(at 12½ days) in a
region of rich mesenchymal vascularity that signals the start of tooth
formation. The association of a rich vascular network in the areas of
dental lamina formation is an interesting phenomenon. GAUNT(1959),
studying tooth formation in the cat, found that the first tangible

indication of tooth formation was the abundant proliferation of blood
capillaries, even before there was any evidence of tooth bud formation.
By observing the distribution of vascular networks, he was even able to
predict the future sites of tooth formation. The real function of the
vascular system, appearing at such an early stage of tooth formation,
is unknown; but the present study could suggest two possible roles.

First, the association of glycogen-containing cells with vascular
endothelia at regions distant to the thickened epithelia(in 12½ days)
would suggest that a contact guidance phenomenon was at work, to guide
the migrating NC population into the site of tooth formation. NODEN(1975)
has previously reported blood vessels as a possible substrate of NC cell
migration in the maxillary processes of chick embryos.

Second, the early proliferation of a vascular network in conjunction
with oral epithelial proliferation could be responsible for the delineation
of tooth forming sites. NC cells migrate in a back to front sequence in
amphibian jaws(DE BEER, 1947), eliciting tooth formation at regular
intervals. The even separation of teeth in lower vertebrates(such as
reptilia) is attributed to the inhibition zones produced by neighbouring
tooth germs(OSBORN, 1971, 1973), all of which are alike(homodont).
However, mammalian jaws have teeth of different shapes(heterodont),
and the formation of teeth along discrete positions corresponding to
incisors and molars(sometimes with an intervening diastema, as in rodents)
must surely be due to selective aggregation of NC cells at predetermined
points. Such a role could be fulfilled by the early proliferation of a
vascular network and/or the dental lamina. Otherwise, there is nothing
to stop the migrating NC cells from interacting with oral epithelia all
along the way, producing one massive fused tooth having a mixture of

incisor and molar crown forms.

The primacy of the oral epithelium in the present study was a
startling discovery, in view of the fact the previous descriptions
of tooth formation have tended to assign a passive role to oral epithe-
lium and at the mercy of inductive ectomesenchyme. This is a mistaken
view, and data from the present study would suggest that the oral epithe-
lium has an inherent ability to behave according to its own genetic
make-up, without need of an inductive stimulation from NC ectomesenchyme.
In fact, the idea of inductive stimulation by NC cells is totally foreign
to the general character of NC cells through out the rest of the body.
Through the whole range of NC derivation, the differentiation of NC
cell types is always the result of inductive influences and microenviron-
mental cues exerted by surrounding tissues(see Review of Literature).
Existing evidence would indicate that pluripotential NC cells migrate
through out the body in search of permissive environments that would
allow the differentiation of the appropriate cell type. Environmental
cues encountered along the way would serve to gradually restrict the
number of developmental options available to a given population of cells.
Therefore, NC cells become odontogenic due to an interaction with
pharyngeal endoderm(HORSTADIUS & SELLMAN, 1946) but the arrest of
migration and condensation into the future sites of tooth formation is
more likely due to an organising influence exerted by the overlying oral
epithelia.

Support for an active role by oral epithelium(in the selective
aggregation of NC cells)is available from studies on another NC population
known to migrate through the maxillary process of the chick. In the deve-
loping cornea, an invasion by NC cells has been shown to occur; but only
in the presence of an extracellular stroma produced by the overlying

epithelium(DODSON & HAY, 1974; HAY, 1978). The ECM in the stroma then serves to influence the differentiation of fibroblasts from the newly migrated NC ectomesenchyme by permissive interaction(MEIER & HAY, 1974).

The concept of an epithelial influence acting to induce NC cell differentiation has also been reported by investigators dealing with other NC derivatives. In each instance, the inductive effect is believed to be mediated by epithelially-derived collagen and GAG, evoking a permissive interaction with NC ectomesenchyme. In scleral cartilage differentiation, NC cells(in the periocular region) are dependent upon a permissive interaction with a non-diffusible substance secreted by the pigmented retinal epithelium(NEWSOME, 1976). In membrane bone for-mation, epithelial products(collagen, GAG) secreted by proliferating mandibular epithelium serve to elicit osteogenesis from NC ectomesenchyme (HALL, 1980; BRADAMANTE & HALL, 1980). In addition, the epithelium has been credited with the role of accumulating NC ectomesenchyme into the area(HALL, 1980). Finally, the permissive influence of the basal lamina in eliciting odontoblast differentiation from NC ectomesenchyme(THESLEFF, 1977, 1978) has already been mentioned.

There is little doubt that the dental epithelium of the bell-stage is capable of secreting collagen and GAG(TRELSTAD & SLAVKIN, 1974; FRANK et.al, 1979). The present study has demonstrated that such a role could begin with the dental lamina primordium at as early as 12½ days during gestation. At 13½ days, a collagen-rich matrix was found underneath the basal lamina, separated from the glycogen-rich cells by a matrix-free zone. Occasionally, collagen fibres were seen passing across a disrupted basal lamina to establish contact with mesenchymal cell processes. It is of course beyond the province of the present study to attribute a causative

role to such an event other than to speculate that it could serve to initiate a permissive interaction ending with odontoblast differentiation at the bell-stage tooth germ. Apart from this, it could also act as a chemotactic factor to attract NC cell invasion.

Finally, it should be stressed that the present study does not discount the possibility of an interactive event occuring between NC ectomesenchyme and the fully formed dental lamina, serving to maintain and even encourage the further development of the tooth bud. All that has been emphasized is that the initial thickening of the oral epithelium is not necessarily the result of an interaction with NC ectomesenchyme but an expression of genetic preprogramming in dental epithelium.

DETERMINATION OF CROWN FORM

There is controversy regarding the determination of crown form during odontogenesis. In the early stages(8-13 days) of tooth formation, the dental epithelium appears to determine crown form and shape when epithelium and mesenchyme of incisors and molars are recombined(DRYBURGH, 1967; MILLER, 1969). KOLLAR & BAIRD(1969) used older material(14-16 days) and found that the dental mesenchyme, when taken from bell-staged tooth germs, was always responsible for the determination of tooth shape and form when the   recombinants were recovered from intra-ocular trans-plantation. These opposing and conflicting results have puzzled subsequent investigators although many would tend to favour the latter view, that is specificity in dental mesenchyme and not in epithelium as far as crown form determination is concerned.

The results of the present investigation, when interpreted in the light of other ultrastructural studies, have made it possible to combine these discrepant views into a single coherent theory. When one acknow-ledges the primacy of oral epithelium in tooth formation, then it becomes obvious that the information for determining tooth form is initially present within the epithelial cells. The morphogenetic infor-mation for a particular crown form, for example the molars, is genetic-ally present within the molar epithelium. Therefore the bias towards epithelial determination of crown form when tissues in the initial stages of odontogenesis are recombined(DRYBURGH, 1967; MILLER, 1969).

The morphogenetic information is subsequently transferred to the dental papilla during odontoblast differentiation in the bell-stage of tooth development. Once determined, the differentiating odontoblasts

would be capable of exerting a directive influence on whatever epithelial
tissue that is apposed to it, causing it to differentiate into ameloblasts
and according to the desired crown form(KOLLAR & BAIRD, 1970 a,b).

The question that immediately arises is - how and when is this
morphogenetic information transferred from epithelium to mesenchyme?
As KOLLAR(1972) has pointed out, the form determining role appears to
lie within the intervening basal lamina and the associated ECM. It has
been demonstrated that basal lamina, made up by Type 4 collagen, GAG
and other glycoproteins, is manufactured by dental epithelium(TRELSTAD
& SLAVKIN, 1974; FRANK et.al, 1979).During tooth formation *in situ*,
the basal lamina is found in intimate contact with the cell processes
of preodontoblasts(THESLEFF, 1977, 1978). A permissive interaction is
known to take place but it is also plausible that the information for
determination of crown form is simultaneously conveyed to the differen-
tiating odontoblasts. Since this is known to occur at bell-stage, the
dental papilla when recombined with 'uneducated' epithelium from an
external source would in turn have the sole jurisdiction in determining
the shape of the tooth. It should be remembered that when epithelium
is recombined with mesenchyme, it is devoid of basal lamina which has
been digested away enzymatically. Thus, the synthesis of fresh basal
lamina by the epithelium, known to occur only under the organising
influences of mesenchyme(THESLEFF, 1978; KARCHER-DJURICIC, OSMAN, MEYER,
STAUBLI & RUCH, 1979), provides the preodontoblasts with the opportunity
to reorientate the direction of epithelial differentiation.

If the above view was true, then dental epithelium isolated from
the mesenchyme before bell-stage would be expected to express some of
its morphogenetic potential. This has been demonstrated both in the

present study(Fig. 57) and also in the study of KOLLAR & BAIRD(1970b, fig. 2E). Support for above concept can also be found in salivary gland morphogenesis, where the form maintaining role of the basal lamina has been established beyond doubt(see review BERNFIELD, 1978). Removal of the basal lamina resulted in the loss of lobular morphology although it is interesting to note that the epithelium was able to regenerate a fresh basal lamina in the absence of mesenchyme(BANERJEE, COHN & BERNFIELD, 1977).

In conclusion, the oral epithelium has been shown to be possessed of morphogenetic capabilities, more than what it usually has been credited with. The primacy of the oral epithelium in odontogenesis is a force to be reckoned with; confirming the suspicions of STEDING(1967) with epithelium generally. When cautiously extrapolated, this concept could form the basis of renewed understanding in the puzzling events that accompany tooth development.

Chapter 7. CRITIQUE OF THE EXPERIMENTAL METHOD
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1) Sample size

Altogether, 4 pregnant dams were sacrificed, yielding a total of
48 explants divided into equal halves of 12½ and 13½ day old tissues.
However, because of the experimental design, the explants were spread
rather thinly, and often the number of explants receiving identical
culture conditions were limited to one or two only(see Table 2 & 3).
Therefore, the conclusions concerning hard tissue formation were
backed by fairly small sample sizes. The 12½ day old explants were
not so badly affected, and inspite of increased uniformity in culture
conditions, the failure by all the explants(24 in number) to produce
teeth is a significant finding.

2) Size of the explants

As has been pointed out under Discussion, the size of the explant at
the start of culture is a crucial factor. Too large an explant could
include, inadvertently, parts of the maxilla already supplied with
pluripotential NC cells(even though the tooth bearing itself is not),
giving rise to tooth formation in culture. Too small an explant might
jeopardise its chances of survival and hence give rise to a negative
result.

As far as it was possible, great care was undertaken in the present
study to standardize the topographical, albeit arbitrary, limits of
the extirpated dental anlagen. Where an explant was considered
inadequate in terms of size, it was discarded and replaced with a
fresh piece of tissue.

3) Age of the explants

Right from the start of the investigation, the problem of differences in biological age and chronological age was acknowledged. This problem was effectively curbed by ultrastructural examination of the zero-time controls, and where glycogen-rich cells were detected in the vicinity of the dental lamina(of 12½ days), the whole litter was excluded from the study. However, there was still the problem of precocious or retarded littermates; and the likelihood of cultivating tissues not equivalent in developmental status to the zero time controls. The only way to bypass this problem would be to examine ultrastructurally one half of the upper jaw while the other half is cultured.

4) Nutritional requirements

The fact that only 75% of the explants(from 13½ day old embryos) were able to form teeth would imply that all the explants were not equally subjected to the same culture conditions. Apart from tissue trauma, minute differences in culture conditions such as depletion of ascorbic acid content could be responsible for the failures. This is especially true for Medium 199 which contains no ascorbic acid at all. As for hard tissue matrix secretion, all the explants bar one failed to achieve complete cytodifferentiation of ameloblasts and this is possibly due to inadequacies of the culture condition.

5) Ultrastructural identification of glycogen

The identification of glycogen has been unequivocal, although fixation procedures would have inevitably caused a degree of morphological alteration. This was evident in fixation procedures using tannic acid-glutaraldehyde, the glycogen not only decreased in quantity, but also in quality when viewed under the electron microscope. Amylase digestion

advocated by some as a means of histochemical identification of glycogen, is at best still a dubious procedure(WATSON, 1958).

6) <u>Ultrastructural identification of ECM material</u>

The identification of hyaluronate and proteoglycans in the present study is based on artefactual criteria and therefore considered preliminary. The full investigation of this phenomenon should include enzyme histochemistry(Streptomyces hyaluronidase, testicular hyaluronidase) and alcian blue staining at critical electrolyte concentrations (SCOTT & DORLING, 1965). The value of cetylpyridinium chloride(CPC) as a GAG precipitant is questionable(RUGGERI, DELL'ORBO & QUACCI, 1975).

Chapter 8. CONCLUSIONS
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1) In C3H mice, formation of the dental lamina primordium commences
   at 12½ days postconception.

2) The migration of NC cells(in the form of glycogen-rich cells) into
   the nascent regions of maxillary molar formation occurs 24 hours
   later, at 13½ days postconception.

3) The significance of the glycogen-rich cells was tested by *in vitro*
   experimentation; and the results would confirm an indispensable role
   as far as tooth formation in organ culture is concerned.

4) When interpreted in the context of other NC studies, it is safe to
   assume that the glycogen-containing cells are of NC origin; confirming
   the long-suspected contribution of NC cells to mammalian odontogenesis.
   Whether they represent a primary or secondary migration wave, it is
   impossible to tell.

5) The primacy of dental epithelium during the initial stages of tooth
   formation has surfaced as the result of the present investigation.

6) The correlation between vascular elements and glycogen-containing
   cells in 12½ day old mesenchyme is strongly suggestive of a contact-
   guidance phenomenon.

7) Regional differences in ECM content and distribution within the
   mesodermal component of the dental anlage is compatible with current
   theories of NC cell migration and differentiation.

8) Dental tissues extirpated at dental lamina stage was for the first
time shown capable of secreting enamel and dentine matrix *in vitro*.
The exact circumstances giving rise to this occurrence are unfortunately
unknown.

9) Current theories of epithelial-mesenchymal interactions in odontoge-
nesis have little experimental bases, and remain unsubstantiated at
the molecular level. This study has called for a revision of a few
widely-held beliefs, including the role of epithelium in tooth
formation.

10) The migration of the neural crest through out the rest of the
mammalian embryo is an unproven phenomenon. Great caution is therefore
required when interpreting the results of tooth germ experimentation
especially when a complete understanding of NC ontogeny is not
available.

Chapter 9. APPENDICES

APPENDIX 1

Processing for light microscopy

The specimens were subjected to the following procedure at a constant
temperature of $37^{o}C$.

1.	70% alcohol	...	...	1 hour
2.	80% alcohol	...	...	½ hour
3.	95% alcohol	...	...	½ hour
4.	95% alcohol	...	...	½ hour
5.	Absolute alcohol	...	...	½ hour
6.	Absolute alcohol	...	...	½ hour
7.	Absolute alcohol	...	...	½ hour
8.	Chloroform	...	...	½ hour
9.	Chloroform	...	...	½ hour
10.	Chloroform	...	...	½ hour

The tissues were then infused with clean paraffin at a constant temperature
of $56^{o}C$.

1.	Paraffin wax(first change)	...	½ hour
2.	Paraffin wax(second change)	...	½ hour
3.	Paraffin wax(third change)	...	overnight.

The tissues were then vacuumed in paraffin wax at $56^{o}C$ for approximately
15 minutes prior to embedding in blocks.

APPENDIX 2

Procedure adopted with the periodic acid-Schiff(PAS) stain

1. Sections 7 micrometers in thickness were placed in water on a
   glass slide.

2. 1% aqueous periodic acid was used to oxidise the sections for 5 minutes.

3. Washed in running water for 5 minutes and rinsed in distilled water.

4. Placed in Schiff's reagent for 10 - 20 minutes.

5. Rinsed three times in 0.5% aqueous sodium metabisulphite(freshly
   prepared) and washed in running water for 10 minutes.

6. Nuclei stained in celestine blue-haemalum sequence.

7. Differentiated in acid alcohol.

8. Washed in running water.

9. Dehydrated in alcohol, cleared in xylene and mounted in
   synthetic resin medium(PIX).

APPENDIX 3

Dehydration procedure for electron microscopy

1. 30% alcohol	...	...	10 minutes
2. 50% alcohol	...	...	5 minutes
3. 70% alcohol	...	...	5 minutes
4. 95% alcohol	...	...	5 minutes
5. Absolute alcohol	...	...	10 minutes
6. Absolute alcohol	...	...	10 minutes
7. Absolute alcohol	...	...	10 minutes

APPENDIX 4

COMPOSITION OF HANK'S BALANCED SALT SOLUTION

Ingredients per 100 ml:

NaCl	0.8	g.
KCL	0.04	g.
$CaCl_2$	0.014	g.
Na_2HPO_4	0.006	g.
KH_2PO_4	0.006	g.
$MgSO_47H_2O$	0.02	g.
Glucose	0.1	g.
Phenol Red	0.002	g.
$NaHCO_3$	0.07	g.

APPENDIX 5

MEDIUM 199 CONCENTRATE(ingredients per 100 ml)

NaCl	8.0 g	Guanine.HCl	0.3 mg
KCl	0.4 g	Xanthine	0.3 mg
$MgSO_4.7H_2O$	0.2 g	Hypoxanthine	0.3 mg
Na_2HPO_4	0.06g	Thymine	0.3 mg
KH_2PO_4	0.06g	Uracil	0.3 mg
$CaCl_2$	0.14g	Pyridoxal.HCl	0.025mg
$Fe(NO_3)_3.9H_2O$	0.72mg	Pyridoxine.HCl	0.025mg
Glucose	1.0 g	Niacine	0.025mg
Adenine Sulphate	0.01g	Niacinamide	0.025mg
l-Arginine. HCl	0.07g	p-Aminobenzoic Acid	0.05 mg
l-Histidine.HCl.H_2O	0.02g	i-Inositol	0.05 mg
l-Lysine. HCl	0.07g	Riboflavin	0.01 mg
dl-Tryptophane	0.02g	Thiamin.HCl	0.01 mg
l-Tyrosine	0.04g	Calcium Pantothenate	0.01 mg
l-Cystine	0.02g	Choline Chloride	0.5 mg
dl-Phenylalanine	0.05g	Biotin	0.01 mg
dl-Methionine	0.03g	Folic Acid	0.01 mg
dl-Serine	0.05g	Calciferol	0.1 mg
dl-Threonine	0.06g	Cholesterol	0.2 mg
dl-Leucine	0.12g	Tween 80	0.015mg
dl-Isoleucine	0.04g	Alpha Tocopherol(PO_4)	0.01 mg
dl-Valine	0.05g	Menadione	0.01 mg
dl-Glutamic Acid.H_2O	0.15g	Ribose	0.5 mg
dl-Aspartic Acid	0.06g	Desoxyribose	0.5 mg
dl-alpha-Alanine	0.05g	Adenylic Acid	0.2 mg
l-Proline	0.04g	Polymyxin B Sulphate	2000 units
l-Hydroxyproline	0.01g	Neomycin Sulphate	2000 units
Glycine	0.05g	Phenol Red	0.02 g
l-Glutamine	0.1 g	Ethyl Alcohol	0.2 ml
$CH_3COONa.3H_2O$	83 mg		

APPENDIX 6

BGJb MEDIUM(FITTON-JACKSON MODIFICATION)

	mg/L		mg/L
$NaH_2PO_4 \cdot H_2O$	90.00	L-Phenylalanine	50.00
$MgSO_4 \cdot 7H_2O$	200.00	L-Proline	400.00
KCl	400.00	L-Serine	200.00
KH_2PO_4	160.00	l-Threonine	75.00
$NaHCO_3$	3,500.00	L-Tryptophan	40.00
NaCl	5,300.00	L-Tyrosine	40.00
Calcium lactate	555.00	DL-Valine	65.00
Glucose	10,000.00	Alpha tocopherol phosphate	1.00
Phenol red	20.00	Ascorbic acid	50.00
Sodium acetate	50.00	Biotin	0.20
L-Alanine	250.00	Calcium pantothenate	0.20
L-Arginine	175.00	Choline chloride	50.00
L-Aspartic acid	150.00	Folic acid	0.20
L-Cysteine $HCl \cdot H_2O$	90.00	Inositol	0.20
L-Glutamine	200.00	Nicotinamide	20.00
Glycine	800.00	Para aminobenzoic acid	2.00
L-Histidine	150.00	Pyridoxal phosphate	0.20
L-Isoleucine	30.00	Riboflavin	0.20
L-Leucine	50.00	Thiamine hydrochloride	4.00
L-Lysine	240.00	Vitamin B_{12}	0.04
L-Methionine	50.00		

APPENDIX 7

Staining procedure for Haematoxylin and Eosin

1. The sections were allowed to dry at 37°C overnight.

2. Double immersion in xylol, 1 minute each time.

3. Double immersion in absolute alcohol, 1 minute each time.

4. Cleansed in 70% alcohol for 1 minute.

5. Cleansed in 50% alcohol for 1 minute.

6. Washed in distilled water for 1 minute.

7. Stained with Harris' Haemalum for 1 minute.

8. Allowed to blue in weak ammonia solution.

9. Differentiated in 1% hydrochloric acid in 70% alcohol.

10. Stained with eosin for 30 - 45 seconds.

11. Dehydrated with 95% alcohol.

12. Double immersion in absolute alcohol, 1 minute each time.

13. Double immersion in xylol, 1 minute each time.

14. Dried overnight at 37°C.

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