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Enzyme histochemical analysis of Meckel's cartilage*

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INTRODUCTION

There is a difference of opinion about the contribution of Meckel's cartilage to the development of the mandible. Reviews of the literature have been presented by Richany, Bast & Anson (1956) and by Melfi (1966). They summarised the opinions of those who believed that the intramandibular (anterior or distal) portion of the cartilage bar was a temporary structure that simply disappeared, and those who believed that it contributed to the ossification of the mandible. Most investigators have favoured the latter opinion, but the manner and degree in which the cartilage participates in bone formation has not been clarified completely.

In studies of the development and growth of the rat mandible (Bhaskar, 1953; Bhaskar, Weinmann & Schour, 1953) it has been reported that Meckel's cartilage, anterior to the intramembranous ossification at 20 days post-insemination, contributes to the future growth of the mandible. In this area, the cartilage shows a typical cartilaginous degeneration similar to that of other cartilages in developing long bones (Akisaka, 1982).

During a degenerative process such as endochondral ossification, Meckel's cartilage cells might be expected to undergo striking changes in both their morphological and functional aspects. As their morphological modifications correlate inevitably with the organisation of cellular organelles they are responsible for cytochemically detectable enzyme activities.

The main purpose of this study was to clarify changes in the distribution of different hydrolytic, metabolic and oxidative enzymes during the formation and resorption of Meckel's cartilage.

MATERIALS AND METHODS

Sprague–Dawley rats from Anticimex AB (Stockholm, Sweden) were used. Male and female animals were housed together overnight in the proportion of two males to three females. Vaginal smears were taken in the morning, and the day when spermatozoa were present in the smears was designated Day 0 of pregnancy. Pregnant dams were housed in plastic cages and kept in 12 hour light/dark cycles with an ambient temperature of 21 °C. Animals were allowed free access to water and standard pellets.

Under diethyl ether anaesthesia, 48 pregnant dams were killed by decapitation. Two hundred and fourteen fetuses were obtained at daily intervals between embryonic

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Days 11 and 22 and were staged for embryological age according to Edwards (1968). After transport in Histocon® (Bethlehem Trading Co., Gothenburg, Sweden) fetal heads were mounted on cryostat chucks and frozen in isopentane (−140 °C) cooled by liquid nitrogen. Cold microtome sections, 8 μm thick, were cut in a cryostat (System Dittes-Duspiva, W. Germany). Sections were cut in the sagittal, horizontal and frontal planes.

The following staining methods were used. For histological analysis, sections were stained with haematoxylin and eosin. Cartilage was localised by alcian blue staining according to Mowry (1956). Frozen sections were incubated according to standardised methods for reduced nicotinamide adenine dinucleotide diaphorase ($NADH_2$-diaphorase, EC 1.6.99; Chayen, Bitensky & Butcher, 1973), non-specific alkaline phosphatase (APase, EC 3.1.3.1; azo dye method of Burstone, 1960), acid phosphatase (AcPase, EC 3.1.3.2; azo dye method of Barka & Anderson, 1965) and lactate dehydrogenase (LDH, EC 1.1.1.27; Heyden & From, 1969). Control incubations were performed in media lacking enzyme substrates.

RESULTS

At the 13th embryonic day the anterior portion of Meckel's cartilage attained its basic shape and position. Histologically, the cartilage was composed of two precartilaginous cell types; small round densely packed mesenchymal cells and differentiated, less densely packed chondroblasts. The two cartilages, one on either side in the mandibular processes, converged and joined at the anterior ends, where they continued anteriorly in the midline as short rostral processes. A small central segment of each cartilage was composed of chondroblasts.

At the 14th embryonic day, each small condensation of chondroblasts had become a cluster of chondrocytes, surrounded by chondroblasts (Fig. 1*a*). Lateral to Meckel's cartilage was the first indication of pre-osseous tissue that contained osteogenic cells, precursors of membrane bone (Fig. 1*a*). This region was the primary mandibular ossification centre, located anterior to the first molar tooth bud. Alcian blue staining revealed the presence of Meckel's cartilage medial to the mandibular ossification centre (Fig. 1*b*). Non-specific alkaline phosphatase activity (APase) was present around the primary mandibular ossification centre, in the tissue between the membrane bone and Meckel's cartilage, halfway around its circumference in the pre-ossification mesenchymal tissue and in the tissue lateral to the mandibular osteoid (Fig. 1*c*). There was no APase activity in Meckel's cartilage. Acid phosphatase activity (AcPase) was most abundant in resorptive areas in the mandibular osteoid. AcPase activity was also seen in the area lateral to the mandibular osteoid, in myogenic areas medial to Meckel's cartilage and also in the chondrocytes of Meckel's cartilage (Fig. 1*d*). $NADH_2$-diaphorase was present in the cytoplasm of the chondrocytes of Meckel's cartilage, in the osteoblasts of the mandibular osteoid and in the myoblasts medial to Meckel's cartilage (Fig. 1*e*). LDH showed the same localisation as $NADH_2$-diaphorase (Fig. 1*f*).

As the mandibular process grew anteriorly, Meckel's cartilage continued to elongate in this direction by interstitial and appositional growth. At the 15th embryonic day, the rostral processes still appeared as condensed mesenchyme. The differentiating cartilage bars continued to mature having acquired a definite perichondrium, enlarged lacunae and a thin matrix. The forming mandible appeared as a thin plate of membrane bone curving around Meckel's cartilage, lateral and inferior to it.

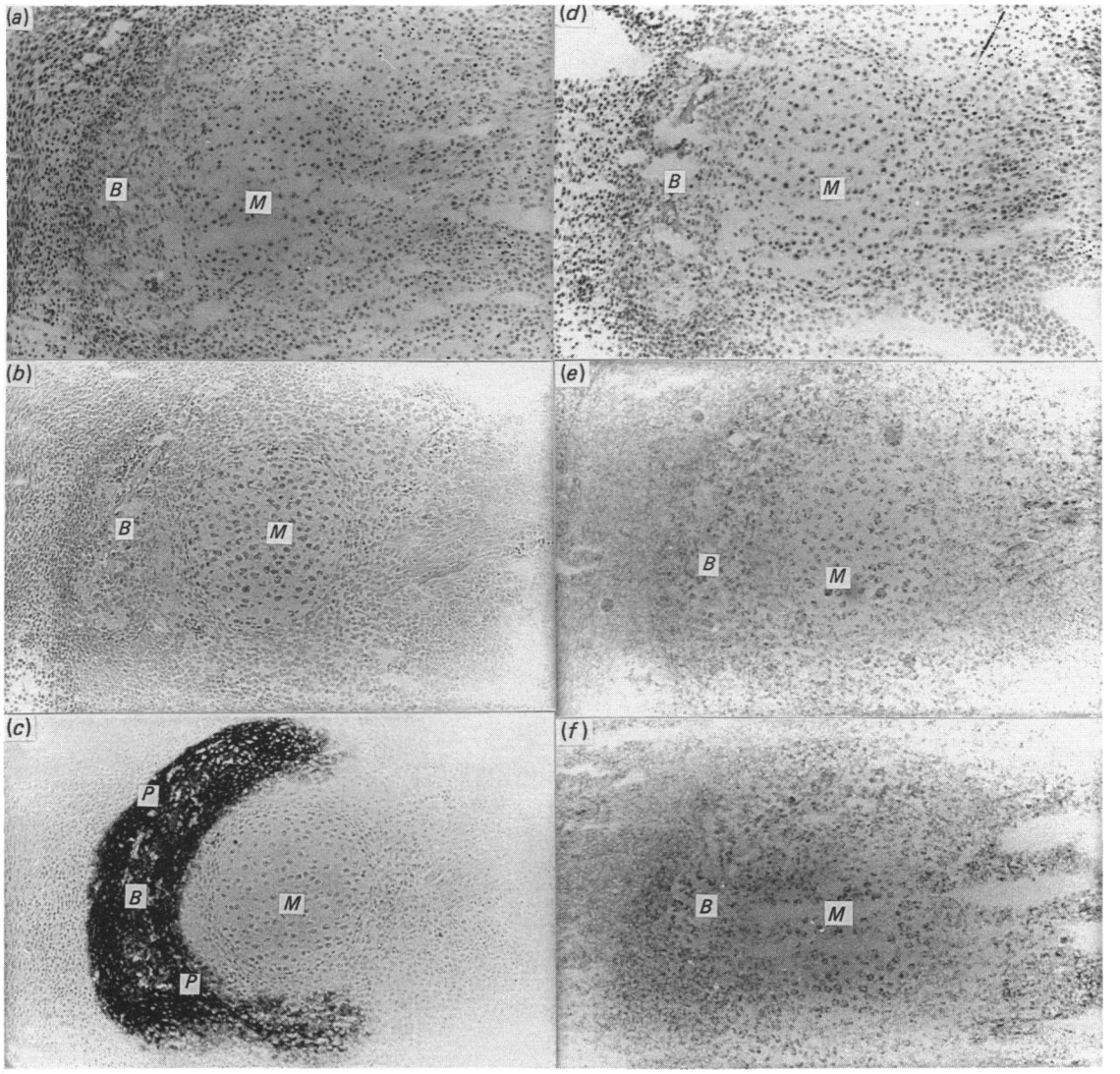

Fig. 1 (*a–f*). Frontal sections through mandibular process at the level of the first molar tooth bud in a 14 days embryonic rat. *M*, Meckel's cartilage; *B*, mandibular ossification centre; *P*, preossification mesenchymal tissue. (*a*) Haematoxylin–eosin, (*b*) Alcian blue, (*c*) APase, (*d*) AcPase, (*e*) $NADH_2$-diaphorase, (*f*) LDH. Original magnification × 80.

At the 16th embryonic day, the anterior portion of each Meckel's cartilage, except for the rostral process was, for the most part, cartilaginous. The adjacent ossified portion of the mandible was more extensive, especially lateral and inferior to the cartilage. The first indication of osteogenic cellular zone formation within the cartilages occurred at this age. The chondrocytes adjacent to the mandibular ossification centre became hypertrophic.

At the 17th embryonic day, a section through the intramembranous ossification centre showed a C-shaped mandible lateral to Meckel's cartilage, with the cartilage occupying the open region. Additional zonal differentiation occurred in Meckel's cartilage anterior to the intramembranous ossification centre. There was a zone of

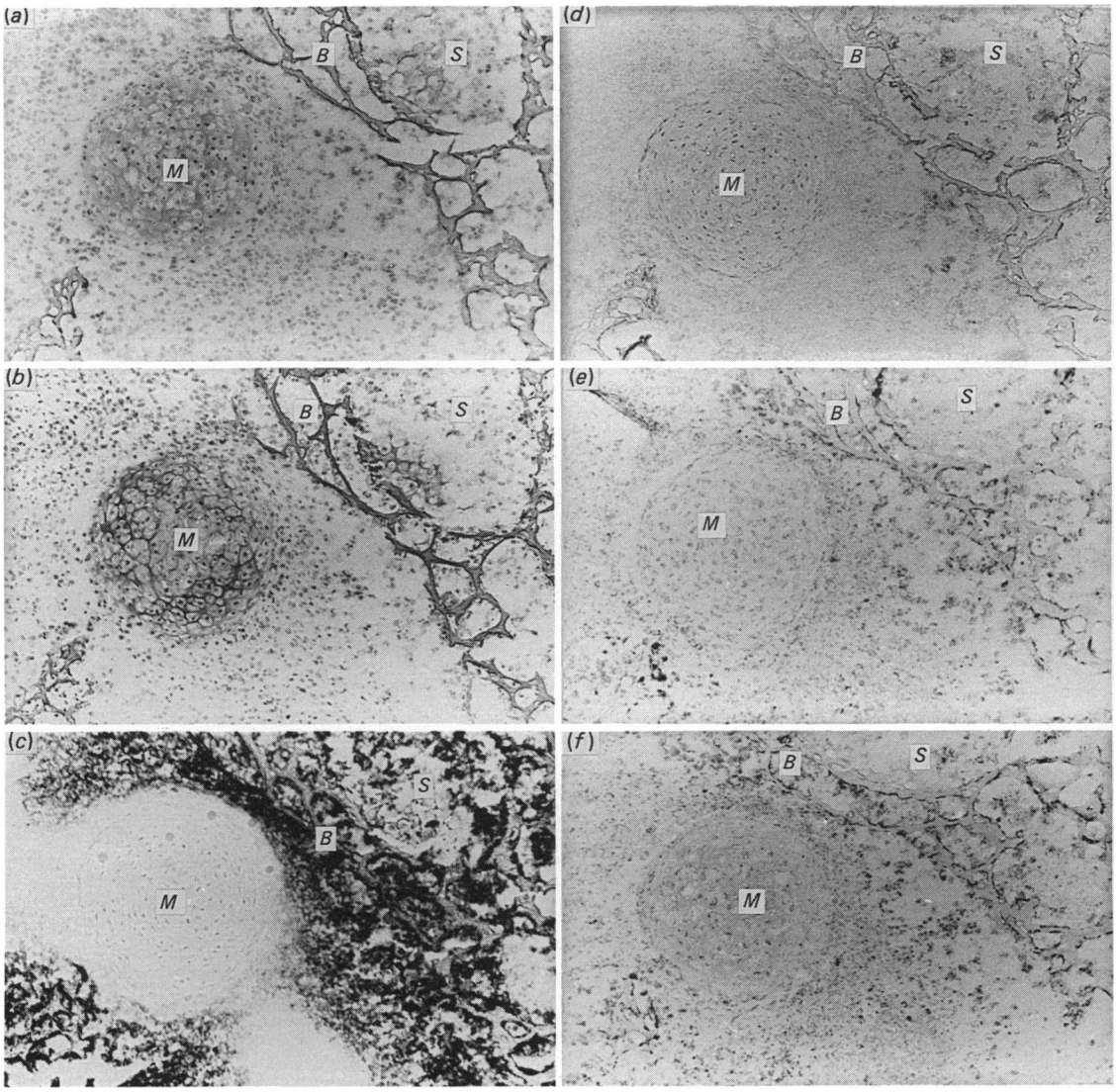

Fig. 2(*a–f*). Frontal sections through plane posterior to the junction of the two cartilage bars (*M*) in 20 days embryonic rat. Secondary cartilage (*S*), membrane bone (*B*). (*a*) Haematoxylin–eosin, (*b*) Alcian blue, (*c*) APase, (*d*) AcPase, (*e*) $NADH_2$-diaphorase, (*f*) LDH. Original magnification × 80.

young cartilage followed by a region of maturing chondrocytes. Adjacent to this was an area of hypertrophic chondrocytes with pyknotic nuclei. As the cells expanded, the matrix between them diminished in quantity and was replaced by a tissue with intense APase activity and a moderate AcPase activity. The endochondral bone that thus formed at the lateral side of Meckel's cartilage filled in the region of the mandible medial to the incisor tooth bud. The trabeculae that developed at the medial side of the cartilage contributed to the lingual plate of the mandible and thus completed its circumference.

At the 18th embryonic day, the membrane bone enclosing Meckel's cartilage extended to the rostral process, which still appeared as condensed precartilaginous mesenchyme. The region of endochondral ossification continued anteriorly with a

thickening and extension of the cellular zones. Posterior to the region of formed endochondral bone at the level of the first molar tooth bud, Meckel's cartilage appeared to be diminished in size and irregular in shape. The matrix in this region was calcified but posterior to this it was scanty, without mineralisation, and it made no direct contribution to ossification of the mandible.

At the 19th embryonic day, the body of the mandible appeared to be almost fully formed. Although intramembranous bone development had progressed further towards the rostrum, this region had not been reached by the advancing endochondral ossification of Meckel's cartilage.

At the 20th embryonic day, the centre of endochondral bone formation progressed anteriorly almost to the junction with the partially calcified bar of cartilage from each side. The ossification zones appeared more definite, particularly the zones of calcification and erosion. At the more osteogenically advanced regions, the cartilage was seen to be split up into several layers with varying degrees of resorption and calcification (Fig. 2*a*). Alcian blue staining revealed a cartilage with large chondrocytes in lacunae but with a periphery of disintegrating cells (Fig. 2*b*). This was accompanied by an intense APase activity in the calcifying matrix (Fig. 2*c*). AcPase activity was present but was more pronounced at the adjacent bone trabeculae (Fig. 2*d*). The $NADH_2$-diaphorase and LDH activities were still weak in the remaining part of Meckel's cartilage (Fig. 2*e*, *f*).

During the early neonatal period, endochondral ossification continued to progress anteriorly along the lateral edges of the bars until it extended as far as the fusion of the two bars at the rostrum.

DISCUSSION

Although it is recognisable in younger embryos as a mesenchymal condensation, the primordium of Meckel's cartilage, as studied in this investigation, is not delineated clearly until the 13th embryonic day. On the 15th embryonic day, the first membrane bone has formed lateral to each cartilage and anterior to the first molar tooth buds. This region of early intramembranous bone formation is the primary mandibular ossification centre. Endochondral ossification becomes apparent on the 17th embryonic day with the mineralisation of the matrix in the adjacent part of the cartilage. The central portions of Meckel's cartilage posterior to this region do not attain the mineralisation stage and are not to be expected to play a role in the ossification of the mandible.

Using grafting experiments, Tyler & Hall (1977) have shown that Meckel's cartilage differentiates either in organ culture or as a graft to the chorioallantoic membrane. After treatment of the mandibular processes with trypsin-pancreatin, epithelium could be removed from the ectomesenchyme, which did however form cartilage. Hall & Tremaine (1979) showed that even before leaving the neural tube, cranial neural crest cells have an ability to chondrify. Bee & Thorogood (1980) have further shown that for this cartilage to form, the neural crest cells have to interact with the epithelial ectoderm, which normally lies adjacent to the neural tube. Thus the ability to form Meckel's cartilage depends on a very early interaction between cells of the cranial neural crest and an epithelium. The membrane bone of the mandible develops because of interactions with mandibular epithelia at their site of differentiation (Tyler & Hall, 1977). Mandibular processes deprived of their epithelium in early stages thus differentiate to form a Meckel's cartilage but not membrane bone. Frommer & Margolies (1971) suggested that Meckel's cartilage rather than mandibular epithelium was responsible for inducing mandibular bone formation. This proposal is

contradicted by the study of Jacobson & Fell (1941), in which mandibular osteogenic centres, isolated at four days of incubation, formed membrane bone in culture in the absence of Meckel's cartilage.

It thus seems that the formation of Meckel's cartilage and the membrane bone of the mandible occurs simultaneously but independently. However, after these structures have formed, they exert considerable interactions on each other.

It is known that differentiated bone cells exist in a vascularised, highly oxygenated environment whereas cartilage cells can survive in an avascular, low oxygen environment (Bassett, 1964). While differentiated cartilage cells are better adapted for existence in an anaerobic environment than the differentiated bone cells, the results of Thorogood & Hall (1976) show that chondrogenic precursor cells are also similarly adapted due to their higher LDH activity. LDH is known to be a key enzyme in anaerobic metabolism, and during late differentiation in rat mandibular processes LDH isoenzymes 3–5 are known to predominate (Granström & Magnusson, 1986*a*), which indicates that this tissue is well adapted for anaerobic metabolism. In cartilage which was under resorption during endochondral ossification, LDH activity had disappeared, indicating a low metabolic activity of this tissue. The osteoblasts on the other hand had a high LDH activity as an indication of high metabolic activity during bone formation. Also in mouse Meckel's cartilage, LDH activity was found from the 15th embryonic day and decreased during hypertrophy of the cells (Heyden & From, 1970).

The high activity of $NADH_2$-diaphorase in the chondrocytes of Meckel's cartilage in addition to a moderate glucose-6-phosphate dehydrogenase activity (Heyden & From, 1970) is evidence of a functional citric acid cycle and pentose phosphate shunt. As $NADH_2$-diaphorase, the LDH activity was present in the chondroblasts and chondrocytes, but diminished during hypertrophy and disappeared during resorption of the cartilage.

We found a moderate AcPase activity in Meckel's cartilage during the whole experimental period. AcPase is considered to be a lysosomal enzyme and is supposed to reduce the proteoglycan content of the matrix (Baylink, Wergedal & Thompson, 1972), which might explain the high activity in the nearby resorbing mandibular bone. The relatively high activity in the cartilage may be explained by the higher proteoglycan content of this tissue.

APase plays a role in biological mineralisation (Granström, 1977). The enzyme consists of four isoenzymes. During embryonic days 14–20 there is a development of isoenzymes, starting from 1 at Day 14, 2 at Day 15 and 3 at Day 16. Isoenzyme 4 appears after birth (Granström & Magnusson, 1986*b*). We found APase activity at its earliest appearance on Day 14 in connection with intramembranous bone formation in the mandibular process. The enzyme activity followed the osteoblasts and osteocytes but also osteoprogenitor cells at a distance from the osteoid. In early phases of Meckel's cartilage development (e.g. Fig. 1*c*), mandibular osteoid and osteoprogenitor cells were seen to envelop Meckel's cartilage on three sides. In this phase of development it is tempting to speculate that Meckel's cartilage actually inhibits bone formation and might therefore have a function as a 'spacer' to keep the centre of the mandible unossified.

It has earlier been shown that Meckel's cartilage in mice contains an additional ATPase (Heyden & From, 1970) and a thiamine pyrophosphatase (TPPase; Akisaka, 1982). ATPase may also participate in the formation of mineralised tissues (Nielsen & Magnusson, 1979), but ATP, the most important energy- and phosphate-containing intracellular component produced by metabolically active cells, is one of the

phosphates that inhibit mineralisation (Fleisch & Neuman, 1961). TPPase is mainly located in the Golgi apparatus. In the chondrocytes, the Golgi apparatus is the site of polysaccharide synthesis and sulphation for matrix formation (Akisaka, 1982). TPPase can be found in the chondrocytes from their formation to the hypertrophic state in Meckel's cartilage of mice (Akisaka, 1982). In human Meckel's cartilage, the chondrocytes have been shown to contain glycogen, a low activity of AcPase, a low activity of non-specific AS-esterase but an absence of APase (Kjaer, 1975).

In conclusion we have found that chondrogenesis of Meckel's cartilage begins at a site anterior to the first molar. Immediately lateral to this locus, the mesenchyme differentiates into the elements of membrane bone formation. Meckel's cartilage shows intense activity of LDH and $NADH_2$-diaphorase and a weak activity of AcPase, whereas the mandibular osteoid showed intense activity of APase. Osteoblasts showed intense activity of LDH and $NADH_2$-diaphorase, and osteoclasts showed intense activity of AcPase. After the differentiation of Meckel's cartilage and mandibular bone, Meckel's cartilage supported mandibular bone formation by endochondral ossification in the anterior part of the mandible.

SUMMARY

Osteogenesis of the body of the mandible in embryonic and neonatal rats was studied histologically and by histochemistry to determine the role of Meckel's cartilage in bone formation. Meckel's cartilage showed intense activity of lactate dehydrogenase and $NADH_2$-diaphorase and weak activity of acid phosphatase, indicating a functioning citric acid cycle, pentose phosphate shunt and a capacity for anaerobic metabolism. The activity of these enzymes declined after hypertrophy of Meckel's cartilage. Alkaline phosphatase was the major enzyme of mineralising mandibular osteoid and was present in the osteoblasts and osteoprogenitor cells but not in Meckel's cartilage. After the differentiation of Meckel's cartilage and intramembranous bone, Meckel's cartilage supported mandibular bone formation by endochondral ossification in the anterior part of the mandible.

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REFERENCES

AKISAKA, T. (1982). The localization of thiamine pyrophosphatase activity in Meckel's cartilage cells during endochondral ossification. *Histochemistry* **76**, 539–546.

BARKA, I. & ANDERSON, P. J. (1965). *Histochemistry: Theory, Practice and Bibliography*, p. 245. New York: Harper & Row.

BASSET, C. A. L. (1964). Environmental and cellular factors regulating osteogenesis. In *Bone Biodynamics* (ed. H. M. Frost), pp. 233–244. Boston, Mass.: Little, Brown.

BAYLINK, D., WERGEDAL, J. & THOMPSON, E. (1972). Loss of polysaccharides at sites where bone mineralization is initiated. *Journal of Histochemistry and Cytochemistry* **21**, 279–292.

BEE, J. & THOROGOOD, P. V. (1980). The role of tissue interactions in the skeletogenic differentiation of avian neural crest cells. *Developmental Biology* **78**, 47–62.

BHASKAR, S. N. (1953). Growth pattern of the rat mandible from 13 days insemination age to 30 days after birth. *American Journal of Anatomy* **92**, 1–53.

BHASKAR, S. N., WEINMANN, J. P. & SCHOUR, I. (1953). Role of Meckel's cartilage in the development and growth of the rat mandible. *Journal of Dental Research* **32**, 398–410.

BURSTONE, M. S. (1960). Histochemical observations on enzymatic processes in bones and teeth. *Annals of the New York Academy of Sciences* **85**, 431–446.

CHAYEN, J., BITENSKY, L. & BUTCHER, R. G. (1973). *Practical Histochemistry*. London: John Wiley.

EDWARDS, J. A. (1968). The external development of the rabbit and rat embryo. *Advances in Teratology* **3**, 239–263.

FLEISCH, H. & NEUMAN, W. F. (1961). Mechanism of calcification: role of collagen, polyphosphate and phosphatase. *American Journal of Physiology* **200**, 1296–1300.

FROMMER, J. & MARGOLIES, M. R. (1971). Contribution of Meckel's cartilage to ossification of the mandible in mice. *Journal of Dental Research* **50**, 1260–1267.

GRANSTRÖM, G. (1977). Alkaline phosphatases in biological calcification. A biochemical study with special reference to ATP-degrading enzyme activity. Thesis, University of Gothenburg.

GRANSTRÖM, G. & MAGNUSSON, B. C. (1986*a*). Lactate dehydrogenase isoenzyme changes during facial development. *Journal of Anatomy* **148**, 183–192.

GRANSTRÖM, G. & MAGNUSSON, B. C. (1986*b*). Changes in alkaline phosphatase isoenzymes of hard-tissue origin during facial development in the rat. *Archives of Oral Biology* **31**, 513–519.

HALL, B. K. & TREMAINE, R. (1979). Ability of neural crest cells from embryonic chick to differentiate into cartilage before their migration away from the neural tube. *Anatomical Record* **194**, 469–476.

HEYDEN, G. & FROM, S. H. (1969). A histochemical study of some oxidative enzymes in mouse molar ontogeny. *Archives of Oral Biology* **14**, 1233–1241.

HEYDEN, G. & FROM, S. H. (1970). Enzyme histochemistry and its application in comparative studies of adenosinetriphosphatase (ATPase) and some oxidative enzymes in bone, cartilage and tooth germs. *Odontologisk Revy* **21**, 129–142.

JACOBSON, W. & FELL, H. B. (1941). The developmental mechanics and potencies of the undifferentiated mesenchyme of the mandible. *Quarterly Journal of Microscopical Science* **82**, 563–586.

KJAER, I. (1975). Histochemical investigation on the symphysis menti in the human fetus related to fetal skeletal maturation in the hand and foot. *Acta anatomica* **93**, 606–633.

MELFI, R. C. (1966). The prenatal development of the human mandible and temporomandibular joint. Thesis, Ohio State University.

MOWRY, R. H. (1956). Alcian blue techniques in the study of acidic carbohydrates. *Journal of Histochemistry and Cytochemistry* **4**, 407.

NIELSEN, R. & MAGNUSSON, B. C. (1979). Enzyme histochemistry of induced heterotopic bone formation in guinea-pigs. *Archives of Oral Biology* **24**, 833–841.

RICHANY, S. F., BAST, T. H. & ANSON, B. J. (1956). The development of the first branchial arch in man and the fate of Meckel's cartilage. *Quarterly Bulletin of Northwestern University Medical School* **30**, 331–355.

THOROGOOD, P. V. & HALL, B. K. (1976). The use of lactate/malic dehydrogenase ratios to distinguish between progenitor cells of cartilage and bone in the embryonic chick. *Journal of Embryology and Experimental Morphology* **36**, 305–313.

TYLER, M. S. & HALL, B. K. (1977). Epithelial influences on skeletogenesis in the mandible of the embryonic chick. *Anatomical Record* **188**, 229–240.