THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

HISTOLOGY AND HISTOCHEMISTRY OF THE EMBRYONIC CHICK MANDIBLE FOLLOWING X-IRRADIATION

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

BY

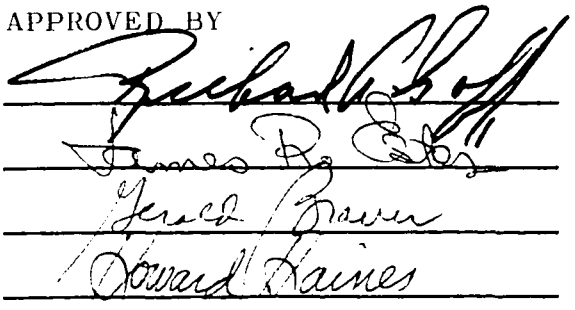
BARBARA ANN BAILEY

Norman, Oklahoma

1970

HISTOLOGY AND HISTOCHEMISTRY OF THE EMBRYONIC CHICK MANDIBLE FOLLOWING X-IRRADIATION

APPROVED BY



DISSERTATION COMMITTEE

ACKNOWLEDGEMENTS

To Richard A. Goff, Professor of Zoology, for the suggestion of and direction of this problem; for the use of his research laboratory and equipment; for his trust, patience, time and encouragement; and for his invaluable assistance in the writing of this dissertation.

To my Committee for their time spent reading and constructively criticising this dissertation.

To Sister Barbara Hansen for her unending help and encouragement received during this study.

To Mr. and Mrs. John R. Wallace and my Parents for their faith, trust and support during this study.

To the many friends who have assisted me in various ways.

To the Department of Zoology for a Graduate Teaching Assistantship and some of the materials used in this research.

TABLE OF CONTENTS

Page

LIST OF TABLES..... vi

LIST OF FIGURES..... vii

LIST OF PLATES..... ix

Chapter

I. INTRODUCTION..... 1

Literature Review
Aspects of the Problem

II. MATERIALS AND METHODS..... 9

X-irradiation Factors
Gross Study
Histology
Histochemistry

III. RESULTS..... 23

Radiation Effects--Gross
Embryonic Deaths
Surviving Embryos--General Effects
Surviving Embryos--Anomaly Characteristics
Histological Observations
Normal Growth Patterns
Normal Cell Death Patterns
Effects of Radiation in the Sensitive Period--General in the Mandible
Effects of Radiation in the Sensitive Period--Cell Death Patterns
Effects of Radiation in the Sensitive Period--Growth Patterns, 1 - 2 Days Post
Effects of Radiation in the Sensitive Period--Growth Patterns, 3 - 4 Days Post

Page

Histochemical Observations
Cell Death Locations
Normal Development Prior to the Sensitive Period, (Stages 18 to 26, 3-4 Days)
Specific Structures in Control and Experimental Mandibles (Stages 27 to 35, 5-9 Days)

IV. DISCUSSION..... 100

Sensitive Period
Anomalous Mandibular Development
Normal Cell Death
Normal Histogenesis
Comparison with Congenital Anomalies
General Comments

V. SUMMARY AND CONCLUSIONS..... 118

LITERATURE CITED..... 120

APPENDIX..... 125

LIST OF TABLES

Table		Page
1.	Acute and Delayed Deaths.....	24
2.	Gross Study--General Information.....	26
3.	Maxilla and Mandibular Reduction.....	28
4.	Maxilla/Mandible Length Ratios.....	30
5.	Cartilage Measurements.....	37
6.	Histochemical Activity Prior to the Sensitive Period.....	68
7.	Histochemical Activity I--Enzymatic.....	71
8.	Histochemical Activity II--Carbohydrate Tests.....	73
9.	Histochemical Activity--Bone Development....	82
10.	Histochemical Activity--Supra-Angular Bone Development.....	83

LIST OF FIGURES

Figure		Page
1.	Typical location of a mandibular blister in a stage 35 (9 day) embryo.....	27
2.	Reduction in mandibular length.....	29
3.	Reduction in maxilla length.....	29
4.	Comparison of maxilla to mandibular lengths at time of sacrifice (625r).....	31
5.	Comparison of maxilla to mandibular lengths at time of sacrifice (700r).....	31
6.	Comparison of maxilla to mandibular lengths at time of sacrifice (800r and 750r).....	31
7.	Cartilage malformations occurring in embryos sacrificed after 9 or 10 days of incubation.....	33
8.	The contour of Meckel's cartilage in a 9-10 day embryo.....	39
9.	Proximal-distal cartilage ratios for mandibles x-irradiated at stages 24, 26+ and 27-.....	40
10.	The mandible at stage 18 (3 days).....	41
11.	The plane in which serial sections of the histological study were cut.....	41
12.	Mandible regions are marked for orientation.....	41
13.	Mandible at sensitive period (stage 27, 5 days).....	43

Figure Page

14. "Spoon" shape of a stage 33 (8 day) mandible..... 43

15. Cartilage and "muscle" relationships in a stage 33 (8 day) mandible..... 43

16. Bone formation in a stage 35 mandible..... 55

17. Mandible at stage 18 (3 days)..... 56

18. Stage 27 (5 days) embryonic mandible..... 56

19. Mandible at stage 30 (7 days)..... 58

20. Cell death pattern II..... 61

21. Cell death pattern III..... 61

22. Diagram of severe mandibular reduction, with a projected normal contour..... 66

23. Mandible at stage 18 (3 days)..... 69

24. Mandible at stage 18 (3 days)..... 69

25. The plane of serial sectioning for the histochemical study..... 69

26. Diagrams of locations of mandibular primordia..... 70

27. Stage 27 mandible (5 days)..... 78

28. Stage 31 mandible (7 days)..... 78

29. Stage 35 mandible (9 days)..... 78

30. Stage 27 mandible (5 days)..... 80

31. Stage 33 mandible (7 1/2 - 8 days)..... 80

32. Stage 35 mandible (9 days)..... 80

LIST OF PLATES

Plate		Page
I.	Gross Study.....	35
II.	Histological Study (A) Hematoxylin-Eosin....	45
III.	Histological Study (B) Hematoxylin-Eosin....	47
IV.	Histological Study (C) Hematoxylin-Eosin....	49
V.	Histological Study (D) Hematoxylin-Eosin....	52
VI.	Histological Study (E) Hematoxylin-Eosin....	64
VII.	Acid and Alkaline Phosphatase Activity (A)..	86
VIII.	Acid and Alkaline Phosphatase Activity (B)..	88
IX.	RNA, ATP and AMP Dephosphohydrolase Activity (A).....	90
X.	ATP and AMP Dephosphohydrolase Activity (B).	92
XI.	RNA, ATP and AMP Dephosphohydrolase Activity (C).....	94
XII.	Carbohydrate Activity.....	96
XIII.	Carbohydrate Activity (B).....	98

HISTOLOGY AND HISTOCHEMISTRY OF THE EMBRYONIC CHICK MANDIBLE FOLLOWING X-IRRADIATION

CHAPTER I

INTRODUCTION

In the development of the chicken beak, the mandible in certain circumstances, may be differentially arrested or deformed. Such a unique localized anomaly appears to be an appropriate subject for the study of morphogenetic mechanisms. Previous workers described genetic mutants (Lamoreux, 1942; Marble, Harper & Hammers, 1944 and McGibbon, 1946) with retarded mandibular development. In addition numerous chemical teratogens cause reduced mandibles (Landauer, 1952, 1953; Stearner, Sanderson, Christian & Tyler, 1960 and Kury & Craig, 1967). X-irradiation also causes anomalous mandibular development (Goff, unpublished data and Stearner, Sanderson, Christian & Tyler, 1960) in chicken embryos.

Previous work dealing with anomalous mandibular development of chicken embryos consisted only of observations that malformations occurred. Developmental stages of chicken embryos are well defined and chicken embryos are easily maintained in relatively simple laboratory facilities. Thus, research was initiated to analyze anomalous mandibular

development using techniques at the gross level and histological-histochemical procedures.

Literature Review[1]

Congenital Mandibular Anomalies

The lower beak consisted of a mere vestige in an anomaly described by Marble, Harper & Hammers (1944). McGibbon (1946) described mandibles seldom more than half the normal length. In extreme cases, the cartilage was bent up or down, almost at right angles to the normal plane. Both mutants were autosomal recessive alleles with the former being lethal in the homozygous condition and the latter semi-lethal.

Chondrodystrophy resulting in an extreme shortening of the lower beak and overgrowth of the upper ("parrot beak") was caused by a homozygous autosomal recessive allele (Lamoreux, 1942). This abnormality was accompanied by shortening of the long bones of the chick.

Chemically Caused Mandibular Anomalies

Landauer (1952) induced abnormalities of the beak with boric acid. The most frequent form was a combination of shortening of the lower beak, cleft palate and bilateral facial coloboma. Each deformity varied in its extent and

---

[1]Unless otherwise stated all citations refer to the chicken embryo.

independent occurrence. Reduced mandibles resulted mainly when treatment occurred between the 84th and 120th hours of incubation. Addition of riboflavin and nicotinamide reduced the teratogenic effects, with riboflavin having the greatest effect. It was postulated that the effects of boric acid may be complex formation with riboflavin linked enzymes.

Injection of 3 mg of pilocarpine (Landauer, 1953) into 95 hr embryos resulted in "parrot beak" and upper beak reduction. Treatment with 6-12 mg caused mainly short lower beaks, suggesting a sensitive period for the mandible. Nicotinamide provided partial reduction in the severity of the anomaly. Such reduction indicated that boric acid and pilocarpine may alter the same metabolic pathways.

Azaserine (O-diazoacetyl-L-serine) injected into the yolk sac of 4 day embryos caused shortened lower beaks, "parrot beaks" and facial coloboma with cleft palate (Dagg & Karnofsky, 1955). Severely shortened mandibles occurred with the least and most severe upper beak defects. Nicotinamide and riboflavin addition did not reduce anomaly formation. It was suggested that azaserine affects a different or later site in the same pathway affected by boric acid and pilocarpine.

"Parrot beaking" with shortened lower jaws was a common effect of eserine sulphate injection between the 2nd and 6th day of incubation (Agarwal, 1956). Maximum sensitivity resulted when the injection occurred on the 4th day.

Edema was often associated with abnormal bending of the lower jaw cartilage and was suggested as a cause of the curvature. Limb skeleton abnormalities also occurred, suggesting a metabolic relationship of facial and limb parts, at least during the sensitive period. It was also suggested that the formation of cartilaginous primordia was altered.

Mitomycin C injection into the yolk sac caused reduced lower jaws when treatment occurred during the 3rd and 4th days of incubation (Kury & Craig, 1967). It is generally considered that mitomycin C inhibits DNA synthesis in intact cells with little initial effect on RNA or protein synthesis.

Radiation Caused Mandibular Anomalies

Most studies using ionizing radiation deal with major abnormalities and their correlation with the stage of irradiation (Clapp, 1964; Goff, 1959; Karnofsky, Patterson & Ridgeway, 1950; and Stearner, Sanderson, Christian & Tyler, 1960). Irradiation between the 2nd and 5th days of incubation resulted in localized accumulations of fluid (Stearner, Sanderson, Christian & Tyler, 1960). Production of fluid filled sacs beneath the mandible, which was often reduced, was a frequent result.

A common response of chicken embryos x-irradiated at 5 days of incubation was hemorrhage in the mandible (Goff, 1959). It was also noted (Goff, unpublished data) that

mandibular reduction resulted.

Cell Death

Cell death in certain locations is a normal event in embryonic development (Bellairs, 1961; Jacobson & Fell, 1941; Glucksmann, 1951; Saunders & Gasseling, 1962 and Whitten, 1968). Mention was made of the occurrence of necrosis in the mid-line of mandibular arches during the period of union of bilateral anlagen (Jacobson & Fell, 1941). Necrotic cells were also noted in pre-osteogenic centers.

Two developmental analyses combining the study of cell death patterns with histochemistry showed that cell death can be responsible for anomaly production (Hinchliffe & Ede, 1967 and Milaire, 1962).

In the study of limb development in the polydactylous talpid^3 mutant of the fowl (Hinchliffe & Ede, 1967), normally occurring cell death, accompanied by the presence of acid phosphatase, was absent from the superficial mesenchyme of the limbs. It was postulated that absence of cell death was responsible for the abnormal sculpturing of the limbs and excessive elongation of the apical ectodermal ridge. Failure of the occurrence of normal necrosis was considered the causative factor in anomaly production.

Oligosyndactylism, a dominant congenital malformation in the mouse, occurs because of fusion between the 2nd and 3rd digits. Gruneberg (1961) suggested that a reduction in mesoderm in the preaxial foot plate was responsible.

Excessive necrosis, with acid phosphatase, was in the preaxial mesoderm of 10 1/2 day limbs (Milaire, 1962) and continued to be present in the limbs of much older embryos. Milaire concluded that massive regional degenerations resulted in reduction of mesoderm during early foot formation.

Histochemistry

There is little histochemistry of even normal embryonic mandibles. The histochemistry of the adventitious cartilage of membrane bones of chick embryos was studied (Hall, 1968). Observations involved the primary cartilage of the mandible only in its relationship to adventitious cartilage. Additional histochemical work with the embryonic beak was primarily concerned with the process of Keratinization (Kingsbury, Allen & Rotheram, 1953 and Yasui & Hayashi, 1968).

Aspects of the Problem

Some gross similarities appear in mandibular anomalies as a result of treatment by teratogens. Effects do not indicate identical responses, but illustrate that the mandible is most sensitive during the 4th and 5th days of incubation and that the cartilage is often abnormally curved.

Radiation, as well as other teratogens, causes unlike effects when applied at different embryonic stages. X-irradiation was selected as the teratogen for this study

because its period of application occurs only during radiation intervals, with possible secondary effects ensuing. The precise time when chemical teratogens reach their destination and cause effects cannot be accurately determined. Timing is critical in elucidating factors of development.

Killing of highly proliferative undifferentiated cells has been established as an early response to radiation. Removal of dead cells, followed by proliferation of remaining cells occurs after this initial damage (Hicks, 1952; Hicks, 1954; and Skalko, 1965). Analysis of cell death localizations before and after x-irradiation enabled determination of primordia most sensitive to radiation, therefore, aiding in resolving causes and evaluation of anomalous formation.

The contribution of Jacobson & Fell (1941) on the developmental potencies of the primitive mesenchyme of the mandible provide an important background for this problem. They determined the location of the cartilage, muscle and membrane bone primordia of the embryonic chick mandible by culturing *in vitro* sections of 3 through 7 day mandibles. They found: (1) Myogenic cells do not originate in the mandibular arches, but migrate laterally from head mesenchyme between the 40th and 60th hr of development and are determined at 3 days. (2) Presumptive cartilage cells are determined at 4 days and originate within the mandibular arches in the proximal mesenchyme and migrate distally as

the muscle primordia move to the mid-line of the mandible. (3) There are two sites of membrane bone primordia, both determined at 4 days; the supra-angular, angular, and opercular bone primordia originate in the proximal mesenchyme and migrate distally; the dentary bone primordium originates in the distal-lateral mesenchyme near its site of initial formation. Basic mandibular primordia are thus determined before the 5th day, i.e., the suggested sensitive period resulting from x-irradiation.

Biochemical events such as the chondrification of Meckel's cartilage begin near the end of the 5th day. Histochemical methods were selected to investigate alterations in biochemical events. By visualizing precise localizations of chemical constituents in developing tissues and detecting some of their enzymatic properties, it was possible to identify some dynamic aspects of normal and abnormal morphogenesis.

This developmental analysis reports the x-irradiation sensitive period of the mandible. Abnormal cartilage contours were determined and correlated with the range of the sensitive period and the various doses applied. Normal and x-irradiation induced necrotic patterns were recorded. Alterations in developing connective tissue, cartilage, bone and musculature were related to the various forms of the anomaly.

CHAPTER II

MATERIALS AND METHODS

White Leghorn eggs for gross and histological studies and some of the histochemical control studies were obtained from the Capitol Hill Hatchery, Oklahoma City, Oklahoma. Eggs for histochemical experiments were obtained from Parkin's Hatchery in Shawnee, Oklahoma and were from a White Leghorn x California Gray stock and from pure White Leghorns. No differences in the x-irradiation effect were detected in the two strains. Storage periods and incubation conditions are summarized on Table A (Appendix).

A forced-draft incubator was used for all experiments. Daily records of temperature and humidity were kept. Eggs were turned three times daily.

X-Irradiation Factors

Some infertility and inviability of eggs prior to utilization occurred (Table B, Appendix). Eggs were generally candled the day of irradiation to eliminate infertile or inviable eggs. Occasionally eggs were candled the day before x-irradiation.

During radiation intervals, the eggs were placed in circular boxes with masonite bottoms and cardboard sides.

The sides were anchored to the bottom by plumbers tape. The boxes were 22.86 cm in diameter and 6.98 cm in height. Foam rubber lined the bottom of the boxes and was molded around a core of plastic foam. Eggs were cushioned with blunt ends toward the periphery of the box. Each box held twelve or thirteen eggs. Eggs were held in position by a lucite cover that rested lightly on their exposed surfaces. A hole in the center of the cover fit over a wooden peg mounted in the center of the box. The cover was held in place by a clamp that grasped the peg. The foam rubber lining made allowance for any size variations so that all eggs were equidistant from the target. Boxes were preheated prior to placing eggs in them.

For x-irradiation the boxes were placed on a turntable within an incubator. The incubator had bottom and sides of masonite and top of 8 mm thick lucite. As the eggs were irradiated, they were rotated at 16 rpm. Incubation temperature was maintained, but humidity was not controlled. The incubator was placed on a table and centered beneath the head of the x-ray machine.

X-irradiation was by a Westinghouse Medium Therapy Unit, with a Machlett CT-250 tube and with inherent filtration of 2 mm of Al. Maximum rating was 250 KV and 15 MA. The machine was operated with added filtration of 1/2 mm Cu and 1 mm Al. Some irradiations were 200 KV and others 250 KV, with no differences detected in the response of embryos.

In order to maintain a constant exposure dose per min, the head to target distance was 52.39 cm at 250 KV and 35.88 cm at 200 KV. Exposure rates ranged from 78r to 86r/min.

Dose was determined with a Victoreen ionization chamber. The chamber was placed in a box identical to those in which the eggs were rotated, except that a special plastic foam platform supported the chamber in a position corresponding to the surface of an egg. Three 1 min readings were taken with the chamber, prior to egg irradiation, and averaged. Readings were corrected for barometric pressure and the characteristics of the ionization chamber. At the end of each experiment, three 1 min readings were repeated. The variation in the before and after readings was never greater than 2 or 3 r. The initial value was taken as the dose rate.

The incubator was located within 3 yards of the x-ray room. The eggs were unincubated during the short period they were carried to the chamber and back. Controls were treated just as the experimental embryos, with the exception that the x-ray machine was not in operation while eggs were rotated. In the gross and the histochemical studies, the number of control eggs was 1/4 to 1/3 the number of experimentals used. Equal numbers of experimentals and controls were used in the histological study. X-irradiation conditions and the stages for x-irradiation and sacrifice are summarized in Tables A and B (Appendix). Following

x-irradiation, eggs were returned to the incubator and not removed from the boxes or turned for a period of at least 12 hr after x-irradiation.

Gross Study

Twenty to twenty-four hr following x-irradiation all eggs were candled to determine acute deaths, i.e., those embryos dying within 24 hr after x-irradiation. These embryos were examined to determine any similarities in their response to x-irradiation. Dead embryos were fixed in neutral buffered 10% formalin (Lillie, 1948). Those embryos undergoing delayed deaths, i.e., those surviving 3 or 4 days after x-irradiation, but dying before the 9th or 10th day of incubation, were also fixed and stored for closer examination. Embryos were considered alive if blood was circulating in the major vitelline vessels. General observations were made at the time of sacrifice. Evidence of hemorrhage, beak deformation and other abnormalities were noted.

Control embryos were staged according to Hamburger & Hamilton (1951). Criteria for staging included: beak and third toe length; presence of protuberance on the posterior side of digit 2 of the wing; presence of labial grooves; number of rows of feather germs dorsal to the eyelid and on the breast; number of scleral papillae; and the position of the nictitating membrane. A stage was assigned for each of these developmental features and an average stage was determined for each embryo. Since the experimental embryos

exhibited a more varied response, they could only be approximately staged.

Measurements of the upper beak length were made from the interior corner of the nostril to the beak tip. The third toe was measured from its tip to the middle of its metatarsal joint. In addition to the standard measurements for staging, each embryo was weighed and the length of the lower jaw was measured. Each formalin fixed embryo was blotted with paper towels prior to weighing. The lower jaw was measured from the corner of the mouth to the tip of the mandible.

A number of embryos in each experiment were set aside for cartilage staining. The head was removed and then stained in Van Wijhts solution for cartilage (Gray, 1952). Embryos were then dehydrated with ethylene glycol monoethyl ether (Cellosolve) and cleared in dimethoxyethyl phthalate (Nelsen, 1945). All cartilage measurements were made using a calibrated ocular micrometer at a magnification of 10x to 20x. Meckel's cartilage was measured from the distal tip to the articular process. When the cartilage was bent or doubled back on itself, each segment was measured and then summed.

Histology

Five-day, or approximately stage 27, embryos were irradiated at doses of 625r and 750r in experiments VIII and IX respectively. Control and experimental embryos were

sacrificed at the following intervals after x-irradiation: 0, 2, 4, 8, 12, 24, 48, 72, and 96 hr. These embryos were fixed in Bouin's solution for 24 hr and then transferred to 70% ethanol for storage. Each embryo was staged and examined for any gross abnormalities. Mandible reductions could be detected in embryos as early as stage 32.

Embryos were then prepared for histological examination. Generally at least two controls and four experimental embryos were selected from each period of sacrifice. Samples from each group were selected so that all types of mandible malformations could be analyzed more closely. Such a selection of mandibular abnormalities could only be done with any degree of assurance when embryos were stage 32 and older. Mandibular blisters could be detected as early as stage 28, so several embryos exhibiting this response were included in each group.

Each embryonic head was removed and trimmed to remove all but a moderate amount of tissue above and below the beak. Heads were then dehydrated and embedded in paraffin.[1] One series of heads was mounted so that long sections of the mandible could be made, while a second series was mounted for cutting sagittal sections. Ten micron sections were cut and mounted on slides with albumin in glycerin. Sections were then stained in Harris' hematoxylin and an aqueous solution of eosin and then dehydrated and cleared. Cellosolve

---

[1]Peel-A-Way, Lipshaw Manufacturing Company.

was used for dehydration and toluene for clearing purposes.

This series of embryos was studied for locations, shapes and relationships of the early cartilage, muscle and bone primordia. Sites of cellular deaths were also noted. Criteria for describing necrosis were those of Glucksmann (1951).

Histochemistry

This study consisted of four experiments (X through XIII), in which groups of embryos at the critical period (stage 27) were x-irradiated and sacrificed serially as in the histological experiments. Embryos were frozen as fresh tissues and not fixed directly. Each embryo was staged, trimmed as for histological preparation, but then placed in a paper ring filled with OCT.[2] The embryos rested upon an object disc on the stage of a CO_2 Quick Freeze Chamber[3] used to freeze the tissue rapidly with a minimum of distortion. The embryo, frozen in a cube of OCT was then either sectioned or removed for storage or re-mounted on an object disc if reorientation was required. It was not always possible to process frozen embryos immediately. When this was the case, frozen embryos were wrapped individually in aluminum foil and stored in a deep freeze at $-20^{o}C$.

---

[2]Tissue-Tek-Mounting Compound, Ames Company, Elkhart, Indiana.

[3]American Optical Company.

In addition to these four experiments, 81 normal embryos were used to perfect the histochemical reactions prior to the x-irradiation experiments. These embryos provided controls additional to those used in the actual experiments. Embryos of stages 18 through 26 were included in these reactions in order to determine enzymatic localizations up to the sensitive period.

Mandibles were sectioned longitudinally with an American Optical Cryostat, model #845. Mandibles were sectioned at: stage 18 through 28 - 15 microns; stage 29 through 33 - 20 microns and stages 34, 35 - 25 microns. Cutting temperatures were between -10 and -20°C.

As each section was cut it was picked up by lightly touching a warm cover slip to its surface. Cover slips were pre-warmed by placement on a grooved aluminum plate that rested on a warming plate. The aluminum plate averaged 37°C. As sections were cut they were placed on the warming plate. In most cases all sections of one mandible remained on the warming plate until the entire mandible was cut.

Mandibles in experiment X were analyzed for non specific acid and alkaline phosphatase. Those in experiment XI were analyzed for activity of AMP phosphohydrolase, ATP phosphohydrolase, ribonucleic acid and deoxyribonucleic acid. Acid mucopolysaccharides were visualized in experiments XII and XIII and the presence of chondroitin sulfate and keratosulfate was checked.

The method of Mulnard (1955) was used for the alkaline phosphatase reactions. Molarity of the substrate, calcium B glycerophosphate (disodium salt, pentahydrate, 0.1% max. L a isomer, Sigma Co.) was 0.019. Extremes of pH were from 8.9 to 9.13 at room temperature. Incubation time varied with age of the embryos. A 2 to 3 hr interval was most suited for embryos at stages 18 through 29, while a 1 to 2 hr interval was optimum for embryos at stages 30 through 35. Incubation temperature was 37°C. Control sections were treated under the same conditions with the exception that substrate was not added to the medium. All sections, post-fixed in 10% neutral formalin + 1% $CaCl_2$ (formal calcium), were dehydrated in cellosolve and cleared in toluene before mounting in permount.

Gomori's (1941) method for acid phosphatase was utilized. Sodium glycerophosphate, the substrate, (disodium salt, 75% isomer, Sigma Co.) was used at a molarity of 0.014. Optimum incubation time intervals were always shorter than those of alkaline phosphatase. With younger embryos the intervals varied from 1 to 2 hr while the oldest embryos required only 30 to 45 min. The substrate was not added to the control medium. pH values ranged from 4.96 to 5.06 at room temperature. Incubation temperature was 37°C. Sections, post-fixed in formal calcium, were mounted on slides with water soluble glycerin jelly.

Adenosine 5' monphosphoric acid (AMP) (Type II, isolated from yeast, 99-100%, Sigma Co.) and adenosine 5' triphosphoric acid (ATP) (from equine muscle, Sigma Co.) were used at molarities of 0.001 and 0.008 respectively in the nucleotide tests. The procedure of Wachstein & Meisel (1957) was used for the localization of these two phosphatases. Values for the pH of the incubation medium for both phosphatases were 7.00-7.1 at room temperature. Incubation time intervals ranged from 45 min to 2 hr for ATP and from 1 hr to 3 hr for AMP, with the younger embryos receiving the longer interval. AMP and ATP were not added to the incubation medium of the control sections. The incubation temperature was 37°C in all cases. Sections of both nucleotide reactions were mounted on slides with water soluble glycerin jelly. All embryos of the phosphatase reactions were post fixed in formal-calcium prior to dehydration and or mounting.

DNA and RNA localization was obtained by using the Methyl-Green Pyronin method of Kurnick (1955). Kurnick recommends the use of pyronin g; however after testing the staining clearness of pyronin y versus pyronin g, pyronin y (Allied Chemicals) appeared to be the better stain and was used exclusively in experiment XI (Methyl green, Hartman-Leddon Co.). All sections were prefixed in formal-calcium for 5 min and then rinsed for 5 min in Chick Ringers'. Control sections were incubated for 30 min at 37°C in a solution

of ribonuclease (Type I-A from Bovine pancreas, Sigma Co.) in glass distilled water (1.0 mg/10 ml). Alternate sections were incubated in glass distilled water. The pH of the solutions ranged from 5.4 to 5.7 at room temperature. No buffer was used in the incubation medium since these solvents may extract proteins and RNA (Opie & Lavin, 1946), thus interfering with the evaluation of the digestion.

After the incubation period, sections were rinsed several times in distilled water and then immersed in the staining mixture for 30 min. Sections were rinsed again in distilled water, immersed in two changes of n-butyl alcohol (5 min each), followed by immersion in two changes of toluene (5 min each). The dehydrated sections were then mounted on slides with permount. Chromatin appeared blue and cytoplasmic RNA pinkish red.

The periodic acid-Schiff (PAS) reaction (Mowry, 1963) was used for the localization of complex carbohydrates. Presence of glycogen was attempted by using diastase (Type V A, from Malt, Sigma Co.) in phosphate buffer (pH - 6) on alternate sections (Barka & Anderson, 1963). Sections were always prefixed in neutral buffered formalin (5 min) and then rinsed in Chick Ringers' (5 min). Alternate sections were then placed in phosphate buffer and phosphate buffer plus diastase and were incubated for a 30 min period at 37°C. Sections were then rinsed in distilled water. In order to remove any aldehyde groups added by the fixative,

an aldehyde blockade was carried out next by placing the sections in a solution of 2,4-dinitrophenylhydrazine (Eastman Organic Chemical) for 10 min. This was followed by a distilled water rinse (5-10 min), and then immersion in a 5% solution of periodic acid for 5 min. Sections were then placed in Schiffs' reagent for 30 min followed by 3 rinses (2 min each) in a sodium bisulfite solution. Sections were then washed and mounted on slides with glycerin jelly.

Several attempts were made to perfect the utilization of diastase in order to localize glycogen. The results are in question and therefore, reference was made only to PAS positive structures.

Chondroitin sulfate and keratosulphate presence was checked by using 0.1% alcian blue (alcian blue 8GN - Matheson, Coleman and Bell) in acetate buffer (pH - 5.6) containing .4 M $MgCl_2$ and .9 M $MgCl_2$ respectively (Scott, Dorling & Quintarelli, 1964). This procedure was carried out at room temperature (24°C).

Hyaluronidase (.1 mg/ ml) (Type I, Bovine Testis, Sigma Co.) in 0.1M phosphate buffer (pH - 6) was used to verify the presence of chondroitin sulfate (Barka & Anderson, 1963). All sections were pre-fixed in neutral buffered formalin and rinsed in Chick Ringers' prior to any staining or incubation. Alternate sections were placed in buffer alone. The incubation time (37°C) was 30 to 60 min depending upon the age of the embryo tested. Sections were then rinsed

several times in distilled water and then placed in a 0.1% solution of toluidine blue for 10 to 30 men. Toluidine blue O powder (Coleman and Bell Co.) had been previously treated to improve its staining quality (Robinson & Bacsich, 1958). Sections were then rinsed for 2 min in a solution containing equal quantities of 1% potassium ferrocyanide and 5% ammonium molybdate. This latter rinse, before dehydration, has been shown to improve the retention of the metachromatic reaction (Bensley, 1934). The rinse was followed by dehydration in two changes of n butyl alcohol (5 min each) and then clearing in toluene (two 5 min intervals). Sections were mounted on slides with permount. Only alcohol stable metachromasia was accepted.

The presence of sulfated and carboxylated polyanions were determined by the procedure of Lev & Spicer (1964). A 1% solution of alcian blue in .1N HCl (pH - 1.14) was used for the presence of sulfated anions and a 1% solution of alcian blue in 3% acetic acid (pH - 2.47) was used for the presence of carboxylated anions. Alternate sections were used in the two tests. Staining was carried out at room temperature (24°C) for 30 min. After staining, those sections at a pH of 1.14 were blotted dry with filter paper, because an immediate rinse with distilled water will raise the pH of the tissues enough so that carboxylated groups will also be stained (Lev & Spicer, 1964). Following blotting the sections were dehydrated in 2 changes of n-butyl

alcohol and cleared in 2 changes of toluene (5 min each). Sections at pH of 2.47 were rinsed immediately in distilled water and dehydrated and cleared. Cover slips were mounted on slides with permount.

Slides were examined with an American Optical Microstar Microscope and a Photomax (Olympus, Tokyo, Japan). Photomicrographs were taken with the Photomax. Kodak Plus X film (35 mm) was used in photographing the histological series, while Kodak High Contrast Copy film (35 mm) was used to photograph the histochemical reactions. A stage micrometer was photographed so that exact magnifications could be determined.

CHAPTER III

RESULTS

Radiation Effects--Gross

Embryonic Deaths

Embryos suffering acute death following irradiation (Table 1) were generally characterized by severe hemorrhages, particularly in the brain and limbs. Blood of the vitelline system often collected in the sinus terminalis. Blood collection in the sinus terminalis was a common occurrence in early embryonic death.

Embryos undergoing delayed death (Table 1) were generally eviscerated and had severe body hemorrhages. Hemorrhages were common throughout the body, with the mesencephalon, cornea, back and thighs being heavily effected areas. The yolk stalk connection near the body was generally constricted with stagnant blood on both sides of the constriction. There was also tendency for blood to collect in the sinus terminalis. Very pronounced mandibular reduction was generally apparent in these embryos, along with digital and limb reduction.

TABLE 1

ACUTE AND DELAYED DEATHS

Exp. No.	Dose (r)	Ave. Stage X-Rayed	No. X-Rayed	No. Acute Deaths	No. Delayed Deaths	No. Surviving	No. With Blisters
Ia	800	26+	36	21	3	12	1
Ib	800	25	33	25	1	7	1
Ic	800	24	33	8	1	24	0
IIa	700	29-	43	22	7	14	7
IIb	750	28	42	27	5	10	3
IIc	750	27	41	17	6	18	8
IIIa	550	27-	46	0	5	41	0
IIIb	625	27-	46	0	4	42	13
IIIc	750	27	24	23	1	0	0
IVa	700	27-	44	29	5	10	2
IVb	665	27-	46	21	5	20	7
IVc	625	27-	45	4	2	39	10
Va	625	29+	36	9	6	21	6
VI	625	24	24	0	3	21	0
VIIa	650	24	11	1	0	9	0
VIIb	700	24	11	2	0	9	0

Surviving Embryos--General Effects

Surviving embryos (Table 2) sacrificed 4 or 5 days following x-irradiation usually had weaker extraembryonic circulations than control embryos.

Embryos surviving doses between 750r and 800r usually had severe brain hemorrhages, as well as hemorrhages on the thighs, wings, cornea and mandibular regions. Hemorrhages occurred in embryos receiving lower doses, but with decreasing frequency, with the mesencephalon remaining the most susceptible location.

Embryonic blisters. A number of x-irradiated embryos had either clear blisters or hematomas beneath their mandibles (Table 1, Fig. 1). There was no correlation between severity of a lower jaw anomaly and presence of blisters. Blisters were often present on embryos that had slight, if any, lower jaw reduction. Of 13 surviving embryos with blisters beneath the mandible (experiment IIIb), 7 were below mandibles with slight reductions in length and 6 were below severely reduced mandibles. No blisters were on embryos receiving 550r. Embryos x-irradiated at 4 days of incubation did not develop blisters. All degrees of mandibular reduction were observed in embryos without blisters.

TABLE 2

GROSS STUDY--GENERAL INFORMATION

Exp. No.	Ave. Stage X-Rayed	No. Staged Prior to Exp.	Dose (r)	X-Irradiated				Controls			
				No.	Ave. Stage Sacrificed	No. of Survivors	Ave. Weight (g)	No.	Ave. Stage Sacrificed	No. of Survivors	Ave. Weight (g)
Ia	26	11	800	36	34-	12(33.3)[a]	0.96	6	35	6(100.0)[a]	1.56
Ib	25	10	800	33	34	7(21.2)	1.10	8	35	7(87.5)	1.72
Ic	24	11	800	33	34	24(72.7)	0.89	10	35	8(80.0)	1.52
IIa	29-	11	700	43	34+	14(32.7)	1.23	12	35+	12(100.0)	1.83
IIb	28	12	750	42	35	10(23.8)	1.42	12	36-	12(100.0)	1.42
IIc	27	11	750	41	34+	18(43.9)	1.26	12	35+	9(75.0)	1.26
IIIa	27-	8	550	46	35+	41(89.1)	1.52	12	35+	12(100.0)	2.23
IIIb	27-	8	625	46	35+	42(91.3)	1.58	12	36	10(83.3)	2.69
IIIc	27	8	750	24	--	0(00.0)	--	24	35-38	23(95.8)	--
IVa	27-	6	700	44	35	10(22.7)	1.46	12	36	10(83.3)	2.45
IVb	27-	6	665	46	35+	20(45.6)	1.64	12	36+	12(100.0)	2.72
IVc	27-	6	625	45	35+	39(86.6)	1.77	12	36	12(100.0)	2.61
Va	29+	12	625	36	35+	21(58.3)	1.80	12	36	10(83.3)	2.40
Vb	29+		235	12	36	10(83.3)	2.24				2.40
VI	24	13	625	24	35+	21(87.5)	1.62	11	36	11(100.0)	2.64
VIIa	24	8	650	11	36	9(81.8)	--	10	36+	10(100.0)	--
VIIb	24	8	700	11	36	9(81.8)	--				

[a] ( ) = % of embryos surviving.

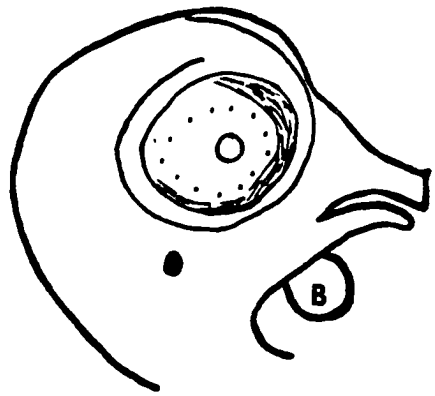


Figure 1. Typical location of a mandibular blister (B) in a stage 35 embryo (9 days).

Sensitive period. The greatest mandibular reduction occurred at 5 days of incubation (approximately stage 27) and this was consistent for all roentgen doses utilized (Table 3, Figs. 2 and 3). Maxilla reduction was much less at corresponding doses, but there was a trend toward greater maxilla reduction at 6 days of incubation (stage 29).

Comparison of maxilla to mandibular lengths as ratios (Table 4) showed that there was very little variation in control values (.43 to .48) while experimental ratios varied considerably (.39 to 1.24). The greatest mandibular reduction occurred when embryos were x-irradiated at 5 days of incubation (Figs. 4, 5, and 6). Control and experimental data (Table 4) were significantly different ($P < 0.10$).

TABLE 3

MANDIBLE AND MAXILLA REDUCTION

Dose	Ave. Stage X-Rayed	No. Controls	No. X-Rayed	Mandibular Length			Maxilla Length		
				Control (mm.)	X-Rayed (mm.)	% Reduction	Control (mm.)	X-Rayed (mm.)	% Reduction
800	24	8	24	3.45	1.80	47.8	1.63	1.23	24.5
800	25	6	7	4.29	1.88	56.2	1.88	1.37	27.1
800	26+	6	12	3.79	1.48	61.0	1.68	1.1	34.5
750	27	9	17	4.52	1.67	63.1	2.16	1.58	26.9
750	28	12	10	4.67	2.39	48.6	2.23	1.46	34.6
700	24	6	9	7.47	4.11	45.0	3.19	2.91	8.8
700	27-	10	10	5.17	1.76	66.0	2.23	1.76	21.1
700	29-	12	14	4.30	1.98	56.3	1.94	1.30	33.0
625	24	9	21	5.22	4.02	23.0	2.37	2.09	11.8
625	27-	20	67	5.13	3.12	39.2	2.27	2.07	8.9
625	29+	10	21	5.06	4.21	16.8	2.41	1.63	32.4

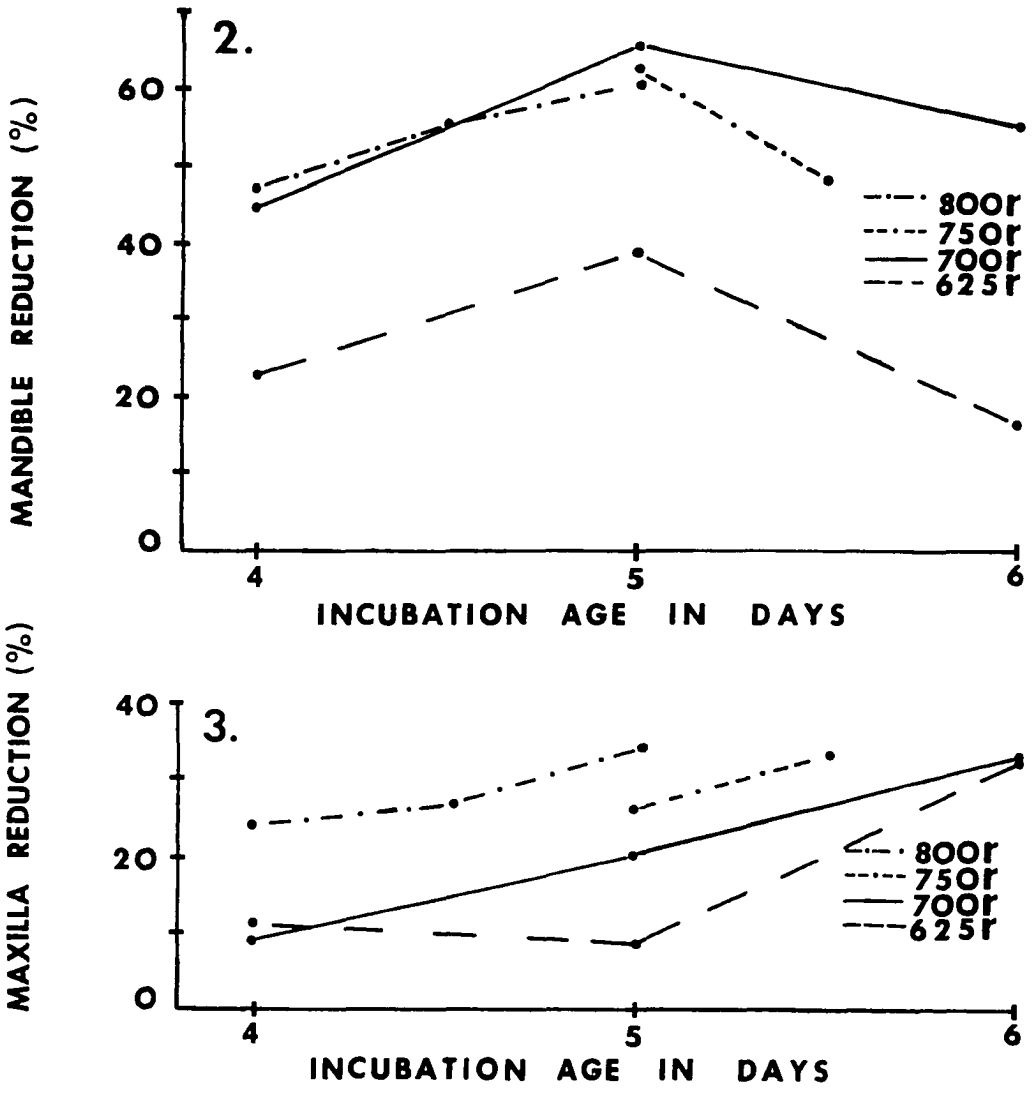


Figure 2. Reduction in mandibular length.

Figure 3. Reduction in maxilla length.

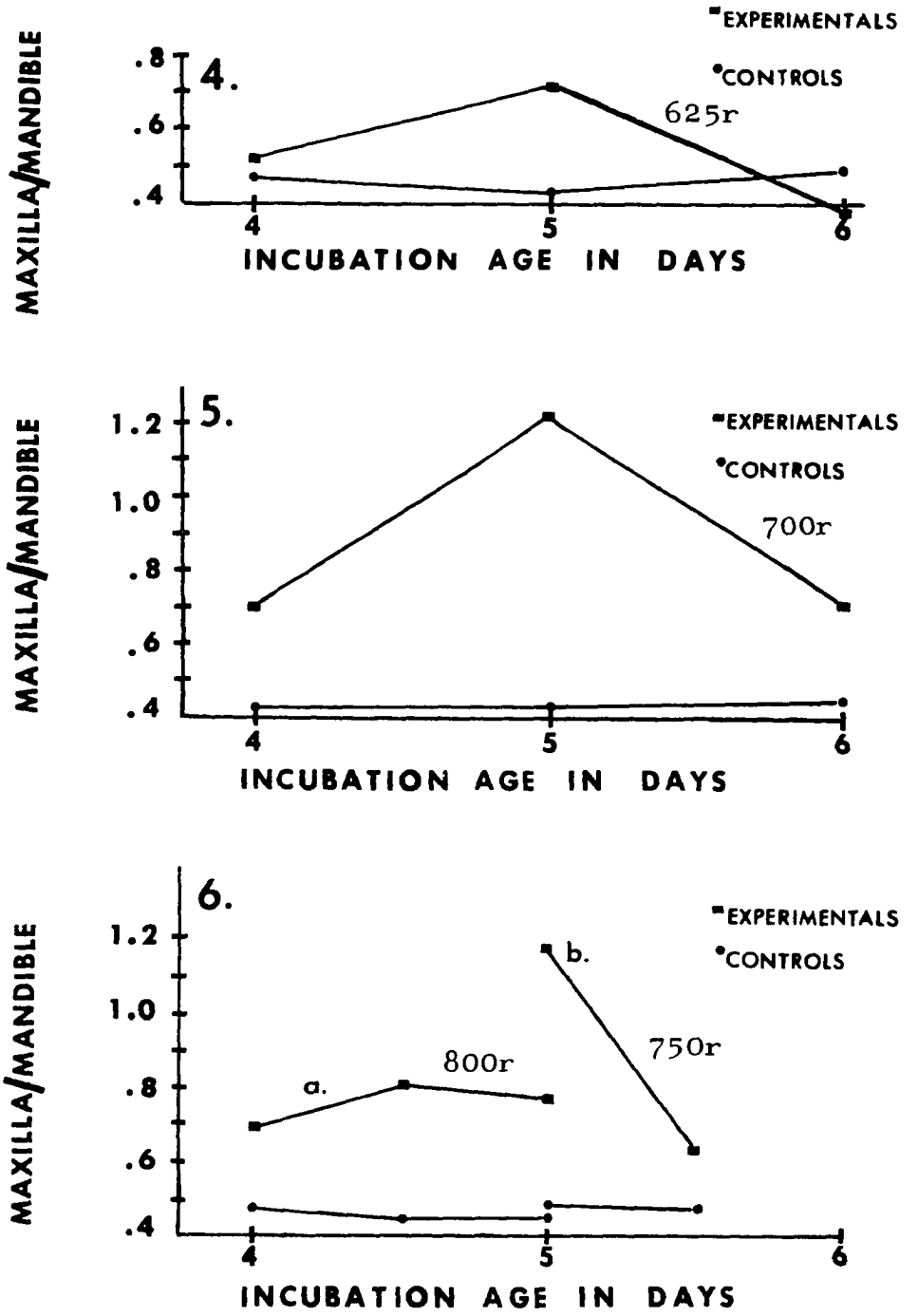
TABLE 4

MAXILLA/MANDIBLE LENGTH RATIOS

Dose	Age		No. Embryos	(CI±s.d.)[a]	(EI±s.d.)[b]	P
	Days	Stage				
800	4.0	24	24	0.47±0.04	0.69±0.15	0.0002
800	4.5	25	7	0.44±0.04	0.81±0.22	0.0020
800	5.0	26+	12	0.44±0.04	0.77±0.26	0.0010
750	5.0	27	18	0.48±0.05	1.17±0.63	0.0005
750	5.5	28	10	0.47±0.03	0.64±0.18	0.0100
700	4.0	24	9	0.43±0.13	0.71±0.25	0.0100
700	5.0	27-	10	0.43±0.52	1.24±0.72	0.0100
700	6.0	29-	14	0.45±0.04	0.72±0.14	0.0001
625	4.0	24	21	0.47±0.07	0.52±0.08	0.0900
625	5.0	27-	81	0.44±0.02	0.72±0.29	0.0001
625	6.0	29+	21	0.48±0.03	0.39±0.05	0.0001

[a]Control index ± standard deviation.

[b]Experimental index ± standard deviation.



Figures 4, 5, and 6. Comparison of maxilla to mandibular length ratios at time of sacrifice at several dose levels.

All forms (to be described) of anomalous mandibles were found in embryos x-irradiated at 5 days. Mandibular anomalies in embryos x-irradiated at 6 days of incubation consisted mainly of moderate reduction in length. Three 6 day embryos stained for cartilage did not exhibit cartilage malformations. Embryos x-irradiated at 4 days of incubation exhibited reduced and curved lower jaws, but not the extreme range of malformations observed in 5 day embryos.

Dose level. A large number of 5 day embryos that received 625r survived (Table 2). This dose was sufficient to cause all variations in the mandibular anomaly. Optimum conditions for the histological--histochemical analysis, therefore, consisted of 5 day embryos irradiated at 625r.

Surviving Embryos--Anomaly Characteristics

Lower jaw malformations varied in degree of reduction and curvature. Figure 7 illustrates the range of malformations of mandibular cartilage (Meckel's cartilage) in order of increasing severity. Severest forms of the anomaly occurred more frequently at 5 days than at 4 or 6 days of incubation. Mandibular reduction increased as the dose increased at all incubation ages, but the peak of mandibular reduction always occurred at 5 days. The maxilla/mandibular ratios were more variable in experimental embryos irradiated at 5 days than in those irradiated at 4 or 6 days, demonstrating the wider range of malformations

Control contour
P-proximal cartilage end
D-distal cartilage end

Typical major bend for embryos x-irradiated at 4 days of incubation.

The Following Contours are for Embryos X-Irradiated at Five Days of Incubation

Typical major bend without an accompaning minor bend.

Incompletely formed minor bend.

Severe doubling back of cartilage in minor bend.

Very severe doubling back of cartilage in minor bend.

Extreme reduction--no bend is evident.

Figure 7. Cartilage malformations occurring in embryos sacrificed after nine or ten days of incubation. Major cartilage bends are the first ones to occur moving from the proximal (P) to the distal (D) end of the cartilage. A minor bend is a second bend occurring within the portion of the cartilage distal to the major bend.

in 5 day embryos.

Several embryos x-irradiated at stage 27 (5 days) were allowed to develop beyond the usual period of sacrifice to illustrate anomaly appearance near hatching age (Plate I, 1a through 1e). Embryo 1a was a control and exhibited the normal conformation of the lower jaw. Cornification began and labial grooves were present on both jaws. Basic forms of the anomaly ranged from slight to extreme mandibular reduction (Plate I, 1b through 1g).

Slight to moderate reduction--no curvature. Comparisons made in the maxilla/mandibular length ratios (Table 4) of a number of experimental embryos, without cartilage malformations, demonstrated a gradation in embryonic response. A small group of embryos had maxilla/mandibular ratios within the range for control embryos. It appeared that the beaks were normal, but the overall size of the embryos had been reduced. Maxilla/mandibular ratios of other embryos did not fall within control values and indicated anomalous development. Mandibular reduction was very small (slight response) for some of these embryos, while the extent of reduction was considerable in others (moderate response). Meckel's cartilage was never buckled or bent in any of these embryos.

Moderate reduction--with curvature. Cartilage curvature with moderate to severe reduction occurred in a large number of embryos (Table 5). Severe reduction with

PLATE I

Gross Study
(Embryos were x-irradiated at stage 27)

1a Control embryo--stage 42--sacrificed 11 days after experimental embryos were x-irradiated. The beak is cornified and labial grooves are well developed. (x 3.5)

1b Experimental embryo--estimated stage 41--sacrificed 9 days after x-irradiation. Mandible is moderately reduced with no curvature. (x 3.8)

1c Experimental embryo--estimated stage 41--sacrificed 10 days after x-irradiation. Mandible is severely reduced with a pronounced curvature. (x 3.7)

1d Experimental embryo--estimated stage 41--sacrificed 11 days after x-irradiation. Mandible is moderately reduced and exhibits a slight bend. (x 3.8)

1e Experimental embryo--estimated stage 41--sacrificed 11 days after x-irradiation. Mandible is severely reduced. (x 3.6)

1f Experimental embryo--estimated stage 35--sacrificed 5 days after x-irradiation. The cartilage exhibits a typical major bend. (x 4.5)

1g Experimental embryo--estimated stage 35--sacrificed 5 days after x-irradiation. A major and a minor bend occurs within the distal end of the cartilage. (x 4.5)

PLATE I

I a

I b

I c

I d

I e

I f

I g

TABLE 5

CARTILAGE MEASUREMENTS

Dose	Stage X-Rayed	Control Length (Mean) mm	Slight to Moderate Reduction[b] (mean) mm	Moderate Reduction[c] Total (Mean) mm	Proximal (Mean) mm	Distal (Mean) mm	Ratio P/D	(S.D.)[d]	No. With Minor Bends	Severe Reduction[e] (Mean) mm
800	24	11.4(3)[a]	7.48(2)[a]	7.2(6)[a]	4.0	3.2	1.3	0.42	--	--
800	26+	11.5(6)	--	6.5(8)	4.2	2.3	2.1	1.09	1	5.17(2)[a]
750	27	14.8(9)	--	10.1(8)	7.4	2.7	2.9	1.23	7	5.66(9)
700	24	11.8(6)	8.8(1)	7.8(5)	3.3	4.5	0.7	0.20	--	--
700	27-	18.7(6)	--	10.8(4)	7.8	3.0	3.0	0.96	4	6.38(2)
665	27-	18.7(6)	12.1(1)	14.2(2)	10.5	3.7	3.0	0.30	2	6.38(1)
625	27-	17.2(16)	13.8(20)	11.3(27)	8.4	2.9	3.2	1.99	15	6.60(4)
625	29	--	6.4(3)	--	--	--	--	--	--	--
550	27-	11.8(6)	8.8(9)	--	--	--	--	--	--	--

[a]( ) = number of embryos.

[b]Reduction without cartilage curvature.

[c]Reduction with cartilage curvature.

[d]Standard deviation.

[e]Without cartilage bends.

marked cartilage bending (Plate I, 1c) and moderate reduction with slight curvature (Plate I, 1d) are illustrated. Cornification occurred in both mandibles.

A major cartilage bend (Fig. 7) was considered the first bend that occurred moving from the proximal (articulation) end of the cartilage to the distal end. Minor bends occurred within the distal portion of a cartilage rod with a major bend. Embryo 1f (Plate I) demonstrates a typical major bend and embryo 1g (Plate I) very severe folding of cartilage in a minor bend. Minor bends were considered part of the distal end of the cartilage when proximal/distal ratios were determined (Table 5). Measurements 9 and 10 day embryos (Table 5) indicated a significant trend for the location of the major cartilage bend to be 2/3 the length of the cartilage from the proximal end, in embryos x-irradiated at 5 days of incubation. Analysis of variance showed high correlation ($P > 0.1$ of a larger F). Minor bends occurred within the distal end. Nine and 10 day control embryos (cartilage stained) were examined for cartilage stress points. The midpoint of the distal curvature occurred within the distal 1/3 of the cartilage (Fig. 8) and most major bends occurred along this distal curve.

Major cartilage bends tended to occur more toward the proximal end of the cartilage in embryos x-irradiated at 4 days of incubation. Cartilage bends for 4 1/2 day

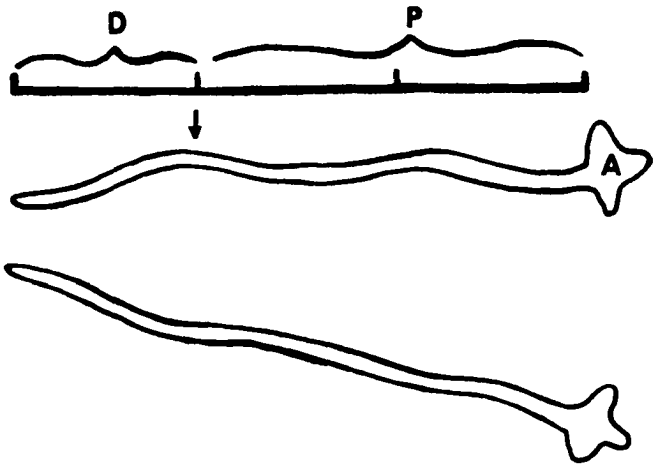


Figure 8. The contour of Meckel's cartilage in a 9-10 day embryo. A--articulation point, D--distal, P--proximal.

embryos occurred between the average bend location in 4 and 5 day embryos. Maxilla/mandibular ratios verify this (Fig. 9).

Extreme reduction--no curvature. In the severest mandibular reduction only a stub of the jaw was present (Plate I, 1e). Meckel's cartilage consisted of short rods not meeting in the mid-line, but protruding as two separate prongs. Average cartilage stub lengths were less than average lengths of the proximal portion of the cartilage in embryos with major bends (Table 5).

Histological Observations

Normal Growth Patterns

Stage 18 (3 days). The mandible was shaped like an archer's bow (Fig. 10) with distinct symphysis in the

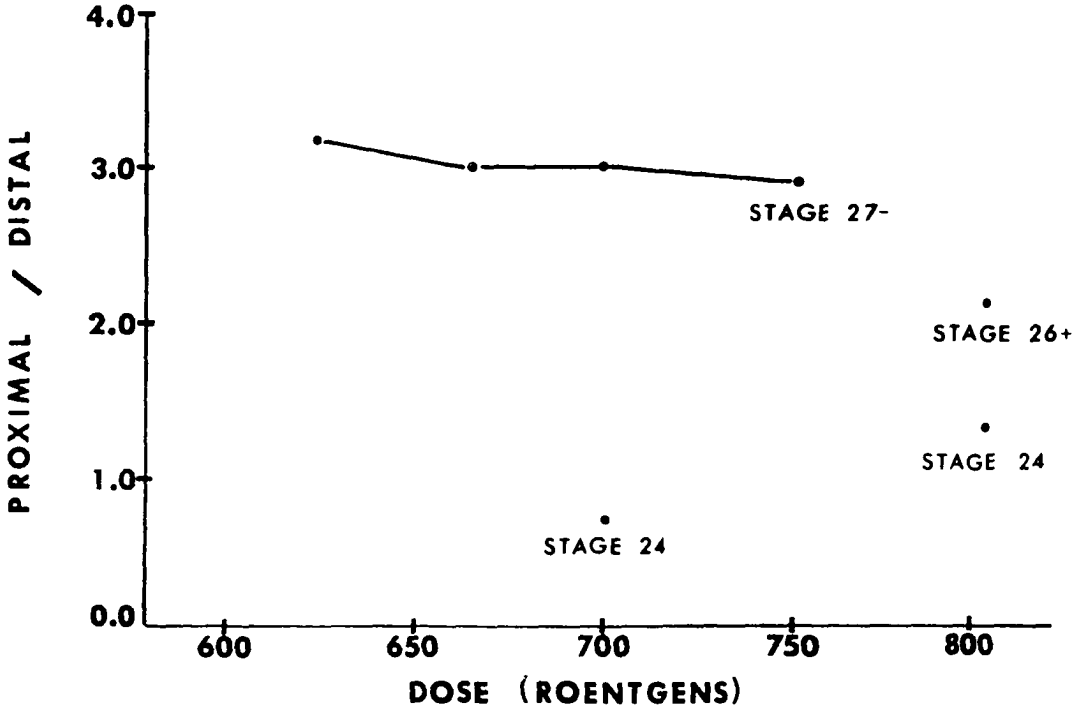
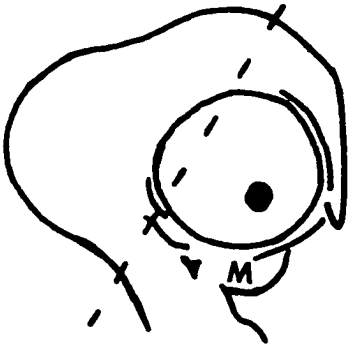
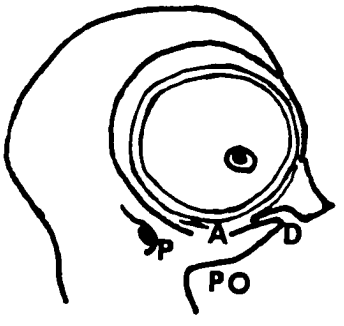


Figure 9. Proximal-distal cartilage ratios for mandibles x-irradiated at stages 24, 26+, and 27-.

10.



11.



12.

Figure 10. The mandible at stage 18 (3 days). T-trigeminal "muscle," N-nerve.

Figure 11. The plane in which serial sections of the histological study were cut. M-mandible (stage 29).

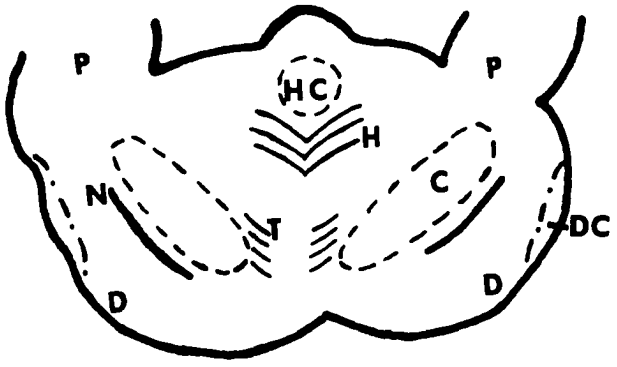
Figure 12. Mandibular regions are marked for orientation (stage 32). A-anterior, D-distal, P-proximal, Po-posterior.

mid-line (see Figures 11 and 12 for orientation). The greatest dimension was in a medial-lateral direction.

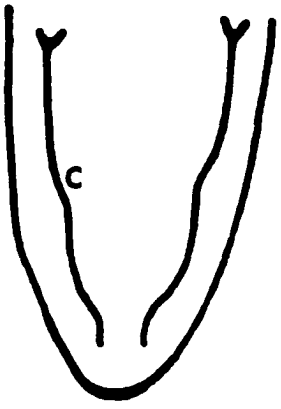
Cell condensations of presumptive muscle were present on either side of the mid-line with nerve fibers of the mandibular rami (of trigeminus nerve) located laterally (Fig. 10). Loose mesenchyme filled the remainder of the mandible. There was no evidence of Meckel's cartilage.

Stage 26, 27 (5 days)--Sensitive Period. Meckel's cartilage primordia formed as two rod-like mesenchymal condensations in the lateral mesenchyme of the mandible (first visceral arches). Nerve fibers were found near lateral condensation surfaces. The quadrates, which form the surfaces for articulation of the lower jaw were present as mesenchymal condensations and were the most anterior structures in the mandible. It was impossible to distinguish a clear separation between the primordia of Meckel's cartilages and the quadrates.

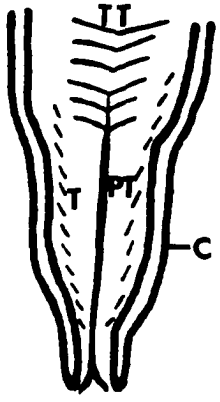
Two sets of presumptive muscle condensations were visible. One set was located medial to the cartilage primordia (Fig. 13), while the second set was found on either side of the mid-line posterior to Meckel's cartilage. Muscle primordia of the mandible will be referred to as trigeminal "musculature." The muscle condensation in the mid-line (hyoid "musculature") was located on the anterior-distal surface of the cartilage primordium of the



13.



14.



15.

Figure 13. Mandible at sensitive period (stage 27, 5 days). C--Meckel's cartilage primordium, D--distal-lateral mesenchyme, DC--dense cell condensation, H--hyoid "musculature," P--proximal mesenchyme, T--trigeminal "musculature," N--nerve, HC--hyoid precartilage.

Figure 14. "Spoon" shape of stage 33 (8 day) mandible. C--Meckel's cartilage.

Figure 15. Cartilage and "muscle" relationship in a stage 33 (8 day) mandible. C--Meckel's cartilage, PT--pretendon, T--longitudinal trigeminal "musculature," TT--transverse trigeminal "musculature."

hyoid skeleton (Fig. 13; Plate II, 2c and 2d).

In addition to cartilage and muscle blastema, a dense cellular condensation was present in the lateral mesenchyme distal to the point of juncture with the maxilla.

Stage 28, 29 (5 1/2 - 6 days). The quadrate was chondrified by the end of the 6th day as indicated by an increase in cytoplasmic basophilia. Meckel's cartilage was chondrifying in a proximal-distal direction with the distal half still unchondrified by the end of the 6th day.

Trigeminal muscle primordia filled most of the area between Meckel's cartilages, which curved medially. Distal cartilage ends were beginning to curve, elongating parallel to one another (Plate III, 3a and 3b). Developing "muscle" was now observed around proximal ends of Meckel's cartilage and will be referred to as "proximal musculature."

Stage 30 - 31 (6 1/2 - 7 days). Distal tips of Meckel's cartilage were nearly parallel. "Muscle" strands (trigeminal) paralleled Meckel's cartilage to the distal tips (Plate IV, 4d). Posterior trigeminal "muscle" developed transversely between the more proximal ends of Meckel's cartilage. Hyoid "musculature" had elongated distally.

Meckel's cartilage was completely chondrified, with the exception of the distal tip (perichondral cap) by the end of the 7th day.

PLATE II

Histological Study (A)--Hematoxylin--Eosin

2a Normal embryo--four days of incubation. The mandibular arch is shown. The mid-line region is the site of considerable necrosis (arrow). (x 49)

2b Detail of the mid-line of the mandibular arch of 2a. Several large macrophages with phagocytosed cells are evident (arrows). (x 371)

2c Control embryo--stage 27. This illustrates the mandibular development at approximately the sensitive period. Necrotic cells may be found along the nerve laterally and in the mid-line, particularly near the distal tip of the cartilage condensation. C--cartilage primordium, N--nerve fibers, T--trigeminal "musculature." (x 49)

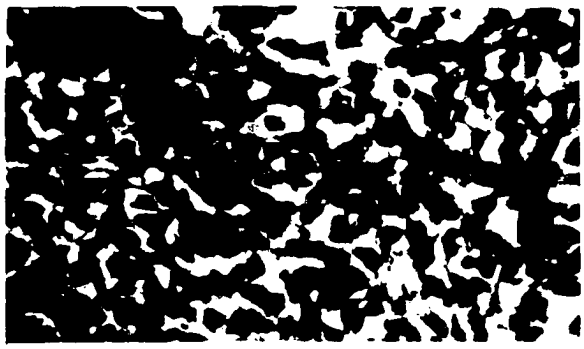
2d Same as 2c. The proximal ends of the precartilage are visible. C--Meckel's cartilage primordium, H--hyoid "musculature." (x 49)

2e Control embryo--stage 27+--sacrificed 30 minutes after experimental embryos were x-irradiated. A very small blister is present at the symphysis of the arch (arrow). (x 49)

2f Experimental embryo--estimated stage 27--sacrificed 2 hours after x-irradiation (dose 750r). A blister is present at the symphysis of the arch. Hemorrhages are evident (arrows). (x 49)

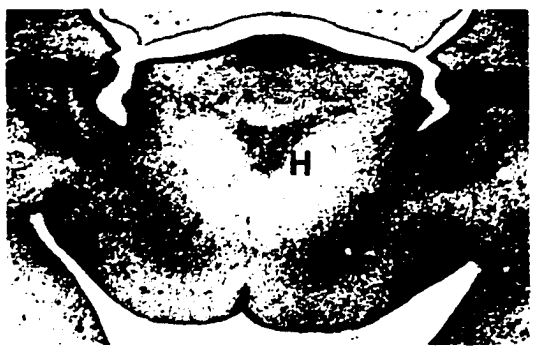
PLATE II

2 a

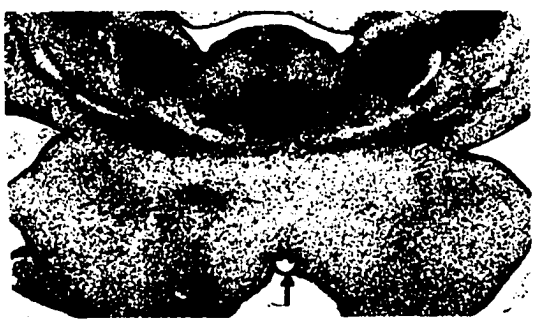


2 b

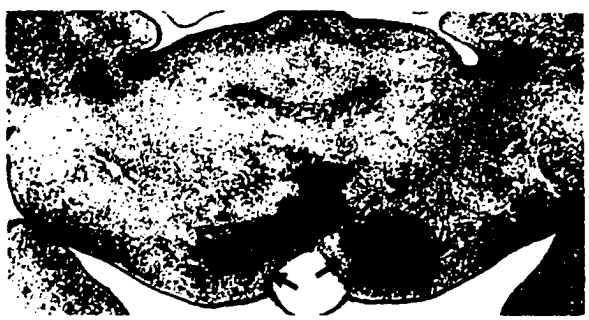
2 c



2 d



2 e



2 f

PLATE III

Histological Observations (B)--Hematoxylin--Eosin

3a Control embryo--stage 29--sacrificed 24 hours after experimental embryos were x-irradiated. Proximal ends of the cartilage are shown. Muscle primordium is growing distally from the developing hyoid apparatus. A strand of developing trigeminal "muscle" can be barely observed running parallel to Meckel's cartilage. B--blood vessel, C--Meckel's cartilage, H--hyoid "musculature," N--nerve, T--trigeminal "musculature." (x 49)

3b Same as 3a. Section is located posterior to that in 3a. Transverse trigeminal "musculature" extends between the proximal ends of Meckel's cartilage. C--Meckel's cartilage, T--trigeminal "musculature." (x 49)

3c Control embryo--stage 27--sacrificed 8 hours after experimental embryos were x-irradiated. Detail of a portion of mandibular nerve running lateral to Meckel's cartilage condensation is shown. A number of necrotic cells (arrows) are located along the borders of the nerve fibers. N--nerve. (x 371)

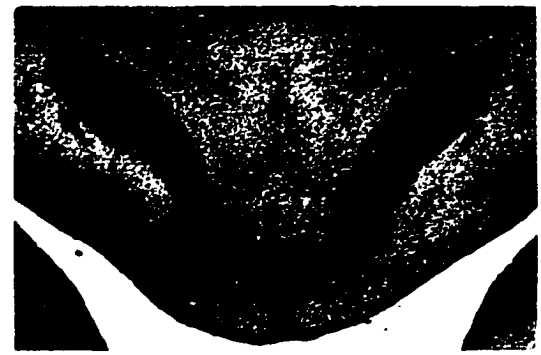
3d Experimental embryo--estimated stage 29--sacrificed 24 hours after x-irradiation. There is evidence of the early cartilage. The trigeminal "musculature" (arrow) along with the perichondral cap is heavily necrotic. C--Meckel's cartilage, T--trigeminal "musculature." (x 49)

3e Same as 3d. This is a greater magnification of the mid-region of the section. Necrotic cells can be distinguished in the mesenchyme and developing muscle (arrow). C--cartilage bend, T--trigeminal "musculature." (x 185)

3f Same as 3d. This section was taken posterior to the cartilage condensations. The tip of a clear blister is visible. S--blister. (x 49)

PLATE III

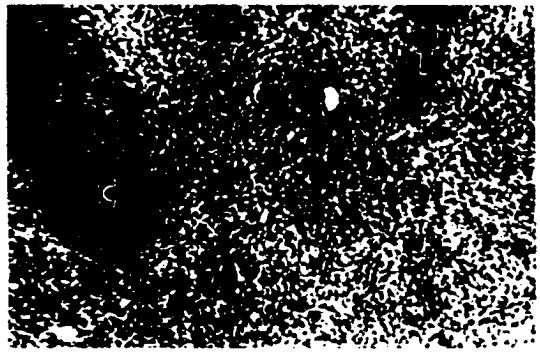
3 a



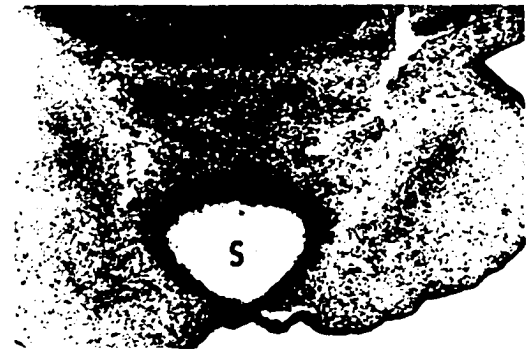
3 b

3 c

3 d



3 e



3 f

PLATE IV

Histological Observations (C)--Hematoxylin--Eosin

4a Experimental embryo--stage 28+--sacrificed 24 hours after x-irradiation. Numerous macrophages are located in the lateral mesenchyme. Some necrosis is evident in the muscle primordia medial to the cartilage and in the mid-line. Muscle primordia have more necrotic figures than the surrounding less dense mesenchyme. C--Meckel's cartilage, H--Hyoid "muscle," T--trigeminal "muscle." Nerve--arrow, LM--lateral mesenchyme. (x 49)

4b Same as 4a. Detail of the right cartilage illustrating the abundant necrotic figures in the lateral mesenchyme. C--Meckel's cartilage, LM--lateral mesenchyme, N--nerve. (x 185)

4c Same as 4a. Detail of the mid-region of the mandibular arch. Necrotic cells are particularly present in the muscle primordia. C--Meckel's cartilage, T--trigeminal "musculature." (x 185)

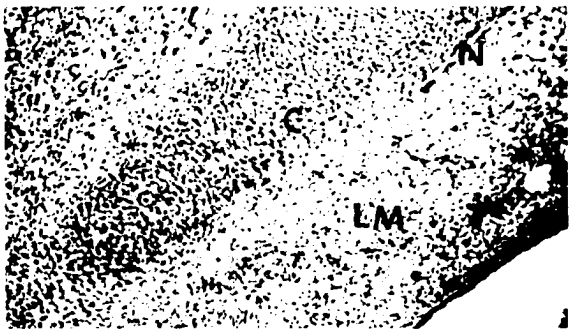
4d Control embryo--stage 31--sacrificed 2 days after experimental embryos were x-irradiated. The distal extent of the cartilage growth is shown. The trigeminal "musculature" extends almost to the distal tip of the cartilage. C--Meckel's cartilage, T--trigeminal muscle. (x 49)

4e Experimental embryo--estimated stage 30--sacrificed 2 days after x-irradiation. Meckel's cartilage has not extended distally as in controls (4d). Mandible has not elongated properly in the proximal-distal dimension. C--Meckel's cartilage, T--trigeminal "musculature." (x 49)

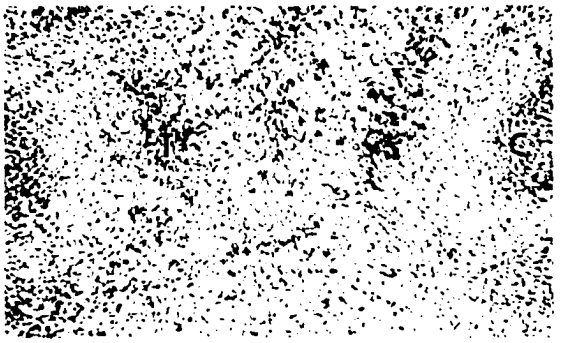
4f Experimental embryo--estimated stage 31--sacrificed 2 days after x-irradiation. Mandible is severely reduced. Little evidence of "muscle" exists and cartilage consists of short stubs. Necrosis is very intense throughout the distal mesenchyme. C--Meckel's cartilage, D--distal mesenchyme. (x 49)

PLATE IV

4 a

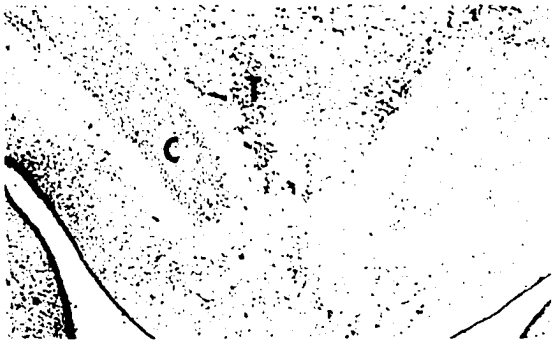


4 b



4 c

4 d



4 e

4 f

Stage 32 - 33 (7 1/2 - 8 days). The mandible was "spoon" shaped (Fig. 14) and Meckel's cartilage was S shaped (Fig. 15). Mesenchyme was dense in the perichondral cap. Narrow bands of trigeminal "muscle" paralleled the cartilage, ending short of the distal tips. (Plate V, 5d). A pre-tendon condensation passed between the cartilage tips (Plate V, 5e) and extended proximally to the transverse trigeminal "muscle." Proximal "musculature" (around Meckel's cartilage articulation) was well developed (Plate V, 5a).

Primordia of the supra-angular and angular bones appeared first as dense cell condensations in mesenchyme lateral to proximal ends of Meckel's cartilage. Ossification began by the 7th and 8th days respectively in proximal portions of each bone (Plate V, 5b and 5c). Cell condensations for the dentary and opercular bones were present by the 8th day in mesenchyme distal-lateral and distal-medial respectively to Meckel's cartilage. The articulation of Meckel's cartilage and the quadrato-jugal bone of the upper jaw, with the quadrate was well established (Plate V, 5a).

Presumptive skeletal structures of the hyoid apparatus were distinct. Hyoid "musculature" along with developing paraglossum extended into the developing jaw (Plate V, 5f).

Stage 34 - 35 (9 days). Relative proportions of

PLATE V

Histological Study (D)--Hematoxylin--Eosin

5a Control embryo--stage 33--sacrificed 3 days after experimental embryos had been x-irradiated. This section was taken through the quadrate-Meckel's cartilage articulation. The proximal "muscles" are well defined. QJ--quadrato-jugal bone, Q--quadrate, C--Meckel's cartilage, P--proximal "muscle." (x 49)

5b Same as 5a. C--Meckel's cartilage, S--supra-angular bone, QJ--quadrato-jugal bone. (x 49)

5c Same as 5a. C--Meckel's cartilage, S--supra-angular bone A--angular bone. (x 49)

5d Same as 5a. The distal extent of the trigeminal muscle is shown between the Meckel's cartilages. C--Meckel's cartilage, T-trigeminal "musculature." (x 49)

5e Same as 5a. Pretendon condensation passes between the distal cartilage tips. C--Meckel's cartilage, PT-pretendon. (x 49)

5f Same as 5a. Tongue development is shown. H--hyoid "musculature," P--developing paraglossum. (x 49)

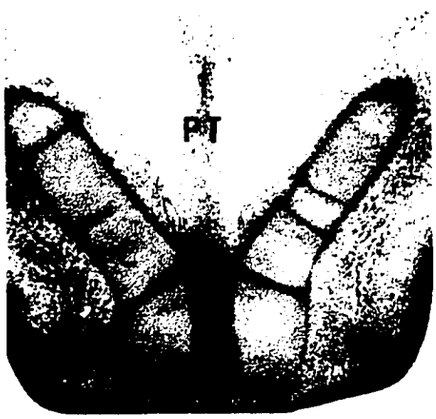
PLATE V

5 a

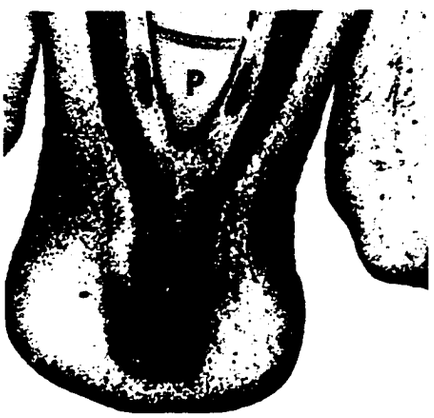
5 b

5 c

5 d



5 e



5 f

Meckel's cartilage and muscle had changed and the trigeminal "muscle" paralleling the cartilage was a comparatively narrow band. Transverse trigeminal "muscle" was a relatively smaller structure. Mandibular nerve rami continued to parallel the lateral surface of Meckel's cartilage.

Opercular and dentary bones appeared as thin plates on the medial and lateral surfaces of Meckel's cartilage respectively. Initial ossification began by approximately 8 1/2 - 9 days in the opercular and dentary bones respectively. Angular and supra-angular bones had well developed trabeculae. Figure 16 is a composite drawing of a series of photomicrographs of the mandible showing respective locations of the 4 membrane bones at stage 35+ (9 days).

Cell condensations indicated the initial appearance of the mentomandibular-gonial bone (endochonral) at the distal ends of Meckel's cartilage.

The tongue was now shaped like a wedge between Meckel's cartilages. Hyoid "musculature" elongated distally as the paraglossum developed.

Normal Cell Death Patterns

Prior to the sensitive period. Necrotic cells, singly or in clumps within macrophages were found in the mid-line of 3 day mandibles (Fig. 17). Isolated necrotic cells were found in lateral regions.

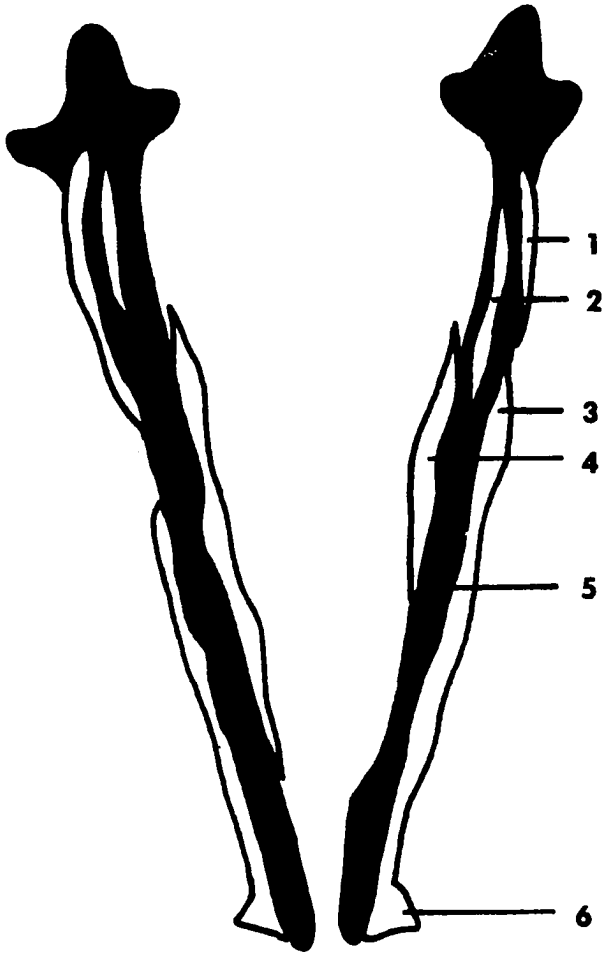
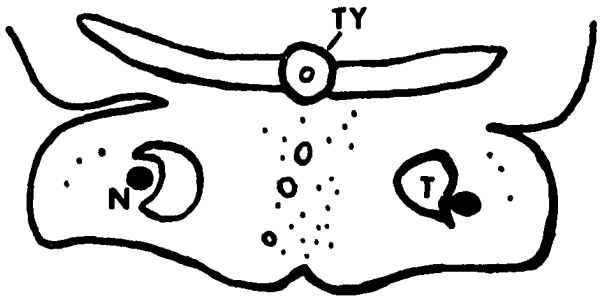
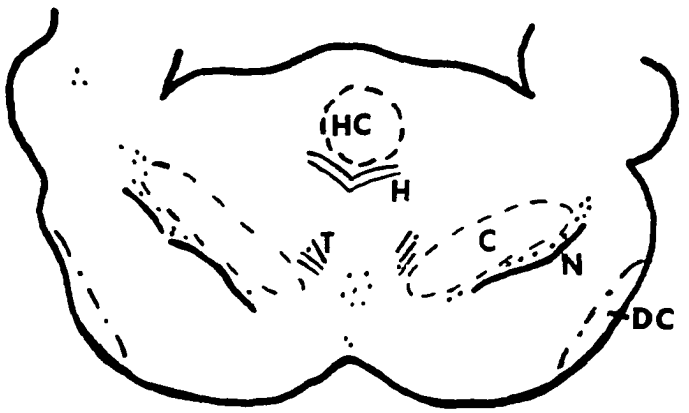


Figure 16. Bone formation in a stage 35 mandible.

1. Supra-angular bone
2. Angular bone
3. Dentary bone
4. Opercular bone
5. Meckel's cartilage
6. Mentomandibular-gonial bone



17.



18.

Figure 17. Mandible at stage 18 (3 days). N--nerve, T--trigeminal "muscle," Ty--thyroglossal duct, .--necrotic cells, o--macrophages.

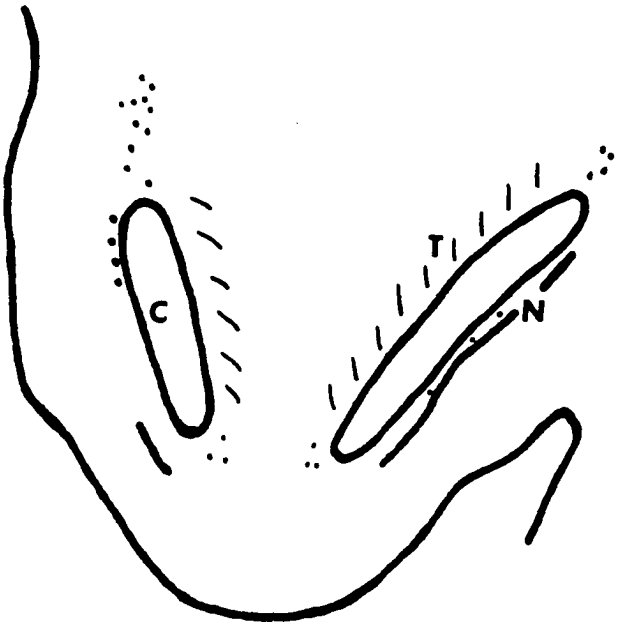
Figure 18. Stage 27 (5 days) embryonic mandible. C--Meckel's cartilage, DC--dense cell condensation, H--hyoid "muscle," HC--hyoid precartilage, N--nerve, T--trigeminal "muscle," .--necrotic cells.

By the 4th day (stages 22 - 24), necrotic cells were nearly all in the mid-mesenchyme of the mandible (Plate II, 2a and 2b). Necrotic cells were present in ectoderm where mandibular arches join the neck of the embryo and in ectoderm of pharyngeal pouches. Near the end of the 4th day, necrotic cells were found along the ramus mandibularis.

The sensitive period. Cell death localizations are shown (Fig. 18). Small clumps of necrotic cells were found on both sides of the most proximal extent of the precartilage and along the entire length of the nerve (Plate III, 3c). Some necrosis was still found in the mid-line, particularly at the distal tip of Meckel's cartilage. Small clear blisters were observed in the mid-line of the symphysis of the arches in several control mandibles (Plate II, 2e).

Later development. Necrotic cells were found lateral to and around the proximal end of Meckel's cartilage through stage 30 (6 1/2 - 7 days) (Fig. 19). During this period, scattered cell deaths were observed near the distal tip of the cartilage and in presumptive hyoid and trigeminal "muscle."

Necrotic cells were also observed around the spinal nerves and around the nerves of the presumptive tongue (hyoid) musculature.



19.

Figure 19. Mandible at stage 30 (7 days). C--Meckel's cartilage, N--nerve, .--Necrotic cells, T--trigeminal "muscle."

Effects of Radiation in the Sensitive Period -- General in Mandible

Cell death--30 minutes--2 hr. post. There was little evidence of cell damage during the interval immediately following x-irradiation up to approximately 30 min. Most striking observation was complete absence of mitotic figures throughout the mandible. Necrosis directly attributed to x-irradiation was evident 1 to 2 hr later.

Cell death--4 to 48 hr. post. Necrotic cells were being phagocytosed by macrophages by 4 hr. after x-irradiation. Patterns of cell death (to be described) remained the same in this period. Macrophages became very abundant from 12 to 24 hr. post. From 24 to 48 hr. post-x-irradiation, the contents of macrophages were generally disintegrating cells (blue-purple dots within macrophage cytoplasm). Nearly all dead cells were cleared out by 48 hr.

Hemorrhages. Embryos receiving 750 r had severe mandibular hemorrhages (Plate II, 2f). Such extreme tissue alterations could not be studied for radiation sensitivity. The bulk of the histological study was therefore on embryos receiving 625r. Occasionally a mandible receiving 625r had a number of small hemorrhages or dilated vessels, particularly in the lateral mesenchyme. One mandible (625r) had a severe hemorrhage in the mid-mesenchyme with a small blister, however, blisters were often present without evidence of hemorrhage.

Mitotic activity. A few mitotic figures were

observed in mandibles by 4 hr. after x-irradiation. Mitotic activity was not conspicuously different than normal by 12 to 24 hr. post x-irradiation in mesenchyme and cartilage primordia. Mitotic activity was observed throughout the entire length of Meckel's cartilage.

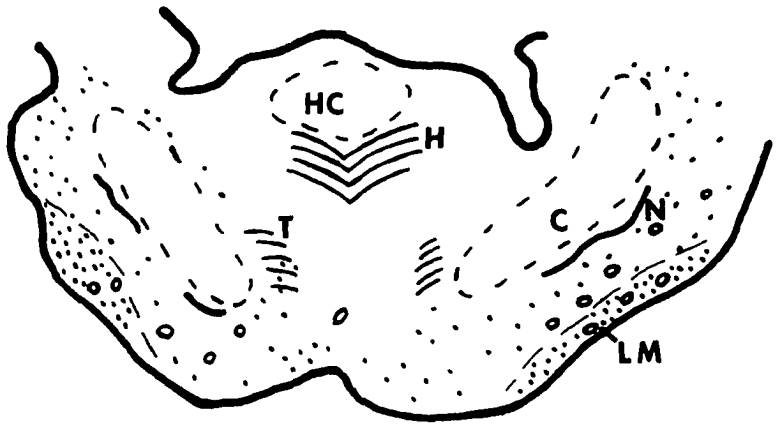
Effects of Radiation in the Sensitive Period -- Cell Death Patterns

A large number of mandibles without excessive hemorrhages exhibited patterns of necrosis which varied depending upon severity of damage.

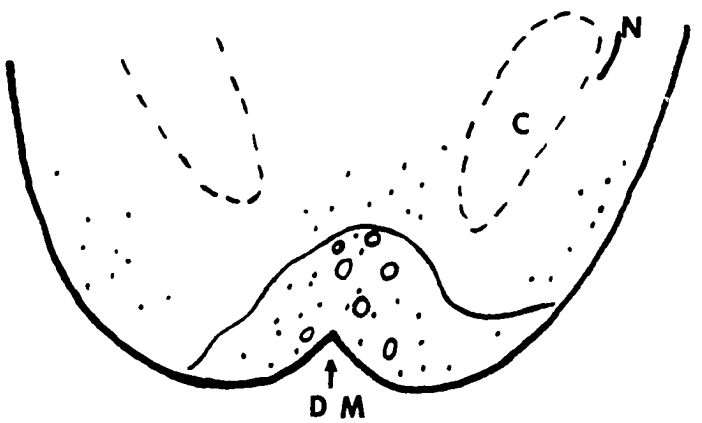
Pattern I. No distinct pattern could be determined for a number of mandibles. Necrotic cells were fairly uniformly scattered throughout the mandible. Radiation damage was not severe.

Pattern II. The lateral mesenchyme was heavily necrotic, with the dense cell condensation of this mesenchyme being the most severely affected. Distal mesenchyme exhibited a lighter degree of necrosis than any of the lateral mesenchyme (Fig. 20; Plate IV, 4a, 4b, and 4c). Pretendon, hyoid and trigeminal "musculature" contained abundant necrotic cells. "Musculature" and pretendon was always more severely affected than the loose mesenchyme present within the mid-mandible.

Meckel's cartilage was not severely affected. Scattered necrotic cells were found throughout the entire length, with no area exhibiting greater necrotic intensity.



20.



21.

Figure 20. Cell death pattern II. (Estimated stage 27-28, 5 1/2 days). C--Meckel's cartilage, H--hyoid "muscle," HC--hyoid precartilage, LM--lateral mesenchyme, N--nerve, T--trigeminal "muscle," .--necrotic cells, o--macrophages.

Figure 21. Cell death pattern III. (Estimated stage 29-30, 6 1/2 days). C--Meckel's cartilage, DM--distal mesenchyme, N--nerve, .--necrotic cells, o--macrophages.

The perichondral cap was more affected than the surrounding loose mesenchyme. Necrotic cells appeared to be fairly evenly distributed in the proximal mesenchyme.

Pattern III. Mandibles had massive necrosis in distal and mid-mesenchyme. Muscle primordia and lateral mesenchyme contained abundant necrotic cells (Fig. 21; Plate IV, 4f). Radiation damage was severe.

Effects of Radiation in the Sensitive Period -- Growth Patterns--1-2 Days Post

Pattern I. Overall size of mandible was reduced, but conformation was normal. Meckel's cartilage and muscle primordia had normal growth patterns.

Pattern II. Meckel's cartilage did not appear to be growing as rapidly as in controls of the same incubation period (Plate IV, 4e). Mandibular contour was blunted in its long dimension. Trigeminal muscle primordia remained more compact and formed a less acute angle (between cartilage rods) than in controls. Some embryos exhibited evidence of bend formation at distal ends of Meckel's cartilage (Plate III, 3d, 3e, and 3f). The mandibular nerve paralleled the cartilage laterally as in controls.

Pattern III. Mandibular development was severely stunted (Plate IV, 4f). Meckel's cartilage consisted of two short stubs. Most of the distal mesenchyme and trigeminal "musculature" were missing. Extensive necrosis

was still present in the distal mesenchyme of several mandibles.

Effects of Radiation in the Sensitive Period--Growth Patterns--3-4 Days Post

Pattern I. Growth Patterns were similar to those of controls.

Pattern II. Proximal "musculature" appeared to be developing normally, although muscle bundles were smaller than in controls. Cartilage bends were evident (Plate VI, 6a, 6b, 6c, 6d, and 6e). Trigeminal "musculature" generally terminated at the initiation, or apex, of a bend. "Muscle" fibers apparently stopped growing distally abruptly in several mandibles. Developing fibers were compacted at this point (Plate VI, 6a). Transversely developing trigeminal "muscle" was more compact than in controls of the same incubation age. Mandibular contours were blunted in their long dimensions. Bent Meckel's cartilages appeared to just fill this contour.

Angular and supra-angular bones were fairly well developed proximally. Opercular and dentary bones were barely present in mandibles sacrificed 4 days post x-irradiation. Bones curved, molding with abnormally curved cartilage. Dentary (and mentomandibular-gonial) bone extended around distal cartilage tips in several mandibles (Plate VI, 6b). Developing bones appeared short and thick.

The tongue was blunted in its long dimension; its

PLATE VI

Histological Study (E)--Hematoxylin--Eosin

6a Experimental embryo--estimated stage 32--sacrificed 3 days after x-irradiation. A bend is present in the left cartilage (note the two pieces). The transverse fibers of the trigeminal "musculature" are present proximally. The trigeminal "musculature" that normally parallels the cartilage ends at the initiation of the bend. The muscle primordia does not appear to have been able to elongate properly (developing fibers are clumped). C--Meckel's cartilage, T--trigeminal "musculature." (x 49)

6b Experimental embryo--estimated stage 35--sacrificed 4 days after x-irradiation. A severe bend exists in the left cartilage. A band of trigeminal "muscle" ends at the point of the bend. The developing dentary and mento-mandibular-gonial bone appears united in the mid-line. C--Meckel's cartilage, D--dentary bone, M--mentomandibular-gonial bone, T--trigeminal "musculature." (x 49)

6c Experimental embryo--estimated stage 31--sacrificed 3 days after x-irradiation. This is a section anterior to the apex of a cartilage bend. Trigeminal "muscle" extends to the initiation of this bend. C--Meckel's cartilage, T--trigeminal "muscle." (x 49)

6d Same as 6c. This section was taken to illustrate the apex of the left cartilage bend. Trigeminal "muscle" extends to this point. C--Meckel's cartilage, T--trigeminal "muscle." (x 49)

6e Same as 6c. The right cartilage bend is shown. The pretendon condensation is visible between the cartilages. C--Meckel's cartilage, PT--pretendon. (x 185)

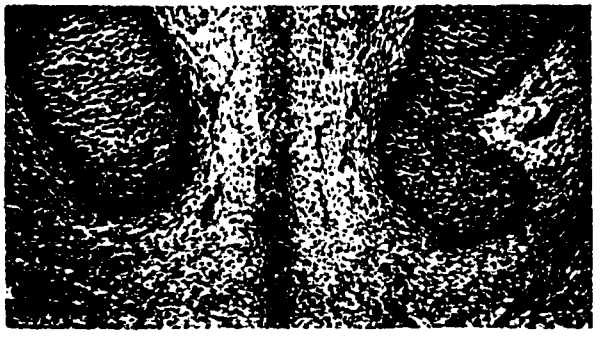
PLATE VI

6 a

6 b

6 c

6 d



6 e

distal and lacked the wedge shaped contour of controls and was bluntly rounded. Hyoid "musculature" was considerably reduced.

Pattern III. No stub mandibles were sectioned at this incubation age. It was apparent after two days of incubation (Fig. 22) that perichondral caps and the bulk of trigeminal "musculature" were destroyed along with the distal mesenchyme.

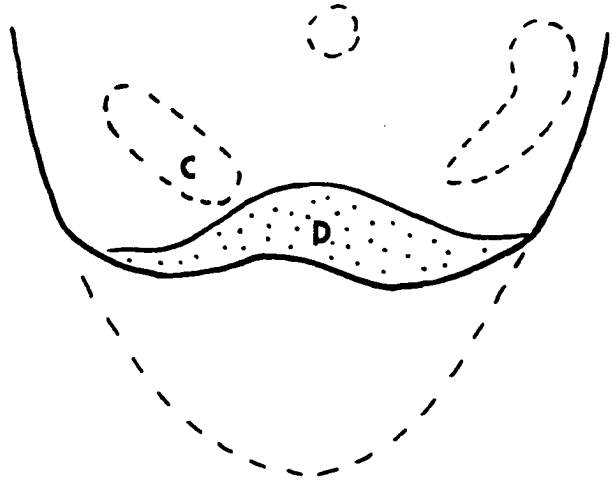


Figure 22. Diagram of severe mandibular reduction, with a projected normal contour. C--Meckel's cartilage, D--distal mesenchyme.

Histochemical Observations

Cell Death Locations

Necrotic cells were sites of intense acid phosphatase activity. Normal and x-irradiation induced cell death patterns were identified, and were consistent with histological observations. In addition to acid phosphase activity in necrotic cells, the mesenchyme surrounding

areas of cell death (resulting from x-irradiation) contained considerable phosphatase activity.

Normal Development Prior to the Sensitive Period (Stages 18 to 26, 3-4 Days)

Semi-quantitative evaluations of the amounts of reaction products in major primordia (Table 6) showed that the ectoderm, nerve and muscle were the main sites of enzymatic activity (Figs. 23, 24) (see Fig. 25 for orientation of sections). Acid (Plate VII, 7a) and alkaline phosphatase and ATP dephosphohydrolase (Plate IX, 9a) exhibited moderate to strong activity in these locations. AMP dephosphohydrolase activity was negative throughout the mandible until stage 26 (Table 6) when moderate activity occurred in the ectoderm and proximal lateral mesenchyme. By stage 26, there was moderate RNA activity in cartilage and muscle primordia (Table 6). In addition RNA and PAS+ were moderate in the ectoderm.

Specific Structures in Control and Experimental Mandibles (Stages 27 to 35, 5-9 Days)

Mandibular sites analyzed in detail are shown in Fig. 26. Cartilage, muscle and bone followed the same growth patterns described for the histology of control and experimental embryos.

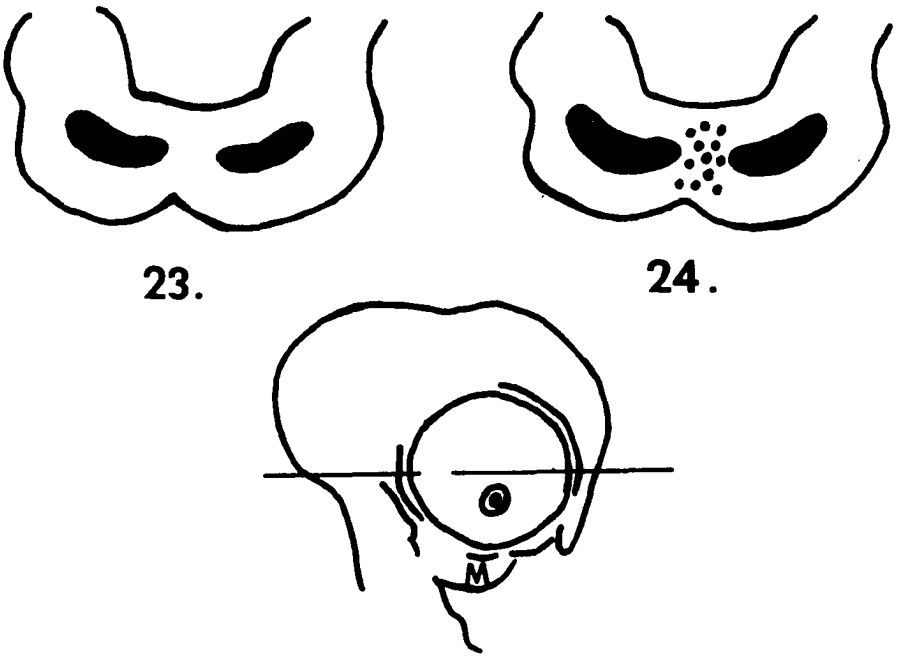
Ectoderm. The dephosphorylating enzymes had uniform activity within the ectoderm, although there was some variation in the amount of activity (Table 7). Alcian

TABLE 6

HISTOCHEMICAL ACTIVITY PRIOR TO THE SENSITIVE PERIOD

Location	Acid P. Stages			Alk. P. Stages			ATP De. Stages			AMP De. Stages			RNA Stage	PAS+ Stage
	18	22	26	18	22	26	18	22	26	18	22	26	26	26
CARTILAGE PRIMORDIA	-	-	-	-	+	+	-	-	+	N	N	N	+	-
MUSCLE PRIMORDIA	++	++	+	+	++	++	++	++	++	N	N	N	+	-
NERVE	++	++	++	+	+	+	+	+	+	N	N	N	N	-
ECTODERM	++	++	++	+	+	+	+	+	++	N	N	+	+	+
PROXIMAL-LATERAL MESENCHYME	-	-	+	-	-	+	-	-	+	N	N	+	-	-
DISTAL-LATERAL MESENCHYME	-	-	+	-	-	-	-	-	+	N	N	N	-	-

KEY: N--Negative reaction
-- Trace or background activity
+ Moderate activity
++ Strong activity
+++ Intense activity
++++ Very intense activity



23.

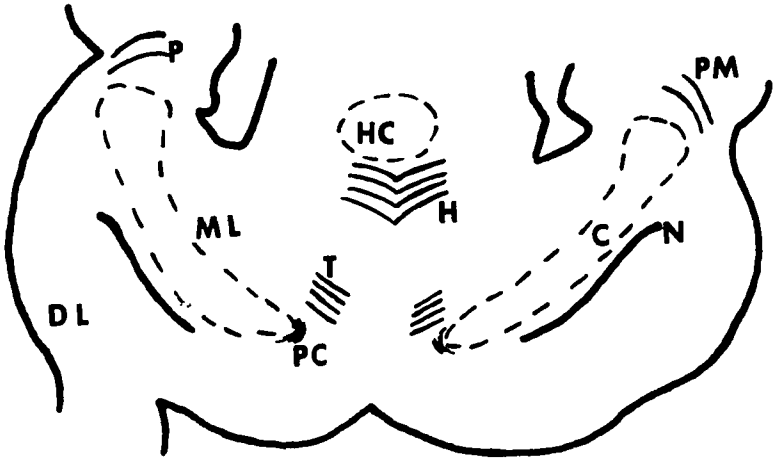
24.

25.

Figure 23. Mandible at stage 18 (3 days). Activity of alkaline phosphatase and ATP dephosphohydrolase is strong in nerve and muscle primordia on either side of the mid-line and in the ectoderm.

Figure 24. Mandible at stage 18 (3 days). Activity of acid phosphatase is strong in nerve and muscle primordia on either side of the mid-line and in the ectoderm. Activity is strong in necrotic cells in the mid-line.

Figure 25. The plane of serial sectioning for the histochemical study. M--mandible.



26.

Figure 26. Diagram of location of mandibular primordia. C--Meckel's cartilage, DL--distal-lateral mesenchyme, H--hyoid "musculature," HC--hyoid precartilage, ML--medial-lateral mesenchyme, N--nerve, P--proximal mesenchyme, PC--perichondral cap, PM--proximal "musculature," T--trigeminal "musculature."

TABLE 7

HISTOCHEMICAL ACTIVITY I--ENZYMATIC

Stages	Acid Phosphatase					Alkaline Phosphatase					ATP Dephospho-hydrolase					AMP Dephospho-hydrolase					RNA				
	27	29	31	33	35	27	29	31	33	35	27	29	31	33	35	27	29	31	33	35	27	29	31	33	35
CARTILAGE	++	++	+	+	+	++	+	-	-	N	+	+	+	-	-	N	-	-	-	N	+	+	++	++	++
PERICHONDRIUM	-	+	+	++	+ ++	-	+	+	-	-	-	+	++	++	++	N	-	+	-	-	-	+	+	+	+
PERICHONDRIAL CAP	-	++	++	++	+	+	+	+	+	N	-	+	++	+	-	N	N	N	N	N	-	+	++	++	-
BONE--SUPRA-ANGULAR	-	-	+	+ ++	+ ++	-	-	+	++	+ ++	-	+	+	++	++	-	+	++	++	++	N	-	+	+	++
MEDIAL MESENCHYME	N	N	-	-	-	-	-	+	+	-	+	+	+	+	+	N	N	N	-	-	-	-	-	-	-
PROXIMAL-LATERAL MESENCHYME	+	+	++	+	+	++	+ ++	++	+	N	+	++	++	++	++	+	++	+	+	-	-	-	-	-	-
NERVE	+	+	+	+	+	+	+	+	-	N	+	+	+	+	+	N	-	-	-	N	N	N	N	N	N
ECTODERM	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	++	++	++
PROXIMAL MUSCLE	+	++	+ ++	+ ++	+ ++	-	+	-	N	N	++	++	+ ++	++	+ ++	N	N	N	-	-	+	+	++	++	+ ++
HYOID MUSCLE	+	+	+ ++	+ ++	+ ++	-	+	-	N	N	+	+	++	++	+ ++	N	N	N	-	-	+	+	+	++	+ ++
TRIGEMINAL MUSCLE	-	+	++	++	+ ++	-	+	-	-	N	+	+	+	++	++	N	N	N	-	-	-	-	+	+	++

Key:
- N Negative Activity
- - Trace or general background activity
- + Moderate activity
- ++ Strong activity
- +++ Intense activity
- ++++ Very intense activity

blue at pH 1.14 appeared specifically in the basement membrane in control and experimental mandibles (Plate XIII, 13f and 13g). Ectoderm was fairly uniformly stained in all other carbohydrate tests (Table 8). There was no difference in the enzymatic activities or carbohydrate staining of control and experimental mandibles.

Muscle development. The proximal and hyoid "musculature" developed more rapidly than the trigeminal "musculature," as indicated by the intensity of the reaction (Tables 7 and 8).

A. Alkaline phosphatase activity appeared during the early development of muscle and diminished (Table 7). By stage 31 (Plate VIII, 8b) there was little activity in muscle primordia.

B. Acid phosphatase activity increased (in muscle fibers) (Plate VIII, 8a) as muscle development proceeded.

C. ATP dephosphohydrolase activity (Plate IX, 9c) increased considerably as fibers began to form. Activity increased progressively from strong to very intense in the proximal musculature (Table 7) (Plates IX, 9f and XI, 11c).

D. AMP dephosphohydrolase activity occurred only in trace amounts during the 7th and 8th days of development (Table 7).

E. RNA increased as fiber development advanced

TABLE 8

HISTOCHEMICAL ACTIVITY II--CARBOHYDRATE TESTS

Position	Alcian Blue[a] Stages				Alcian Blue[b] Stages				Toluidine Blue Metachromasia Stages				PAS+ Stages			
	27	29	32	35	27	29	32	35	27	29	32	35	27	29	32	35
CARTILAGE	+	++	+++	+++	+	++	++	+++	+	++	++	++	+	++	++	++
PERICHONDRIUM	-	+	+	+	-	+	+	++	+	+	+	++	-	+	++	++
BONE-SUPRA-ANGULAR	N	N	-	+	N	N	-	++	N	N	N	N	N	N	+	++
NERVE	N	N	N	N	N	N	N	N	N	N	N	N	-	+	+	+
ECTODERM	+	+	+	+	++	++	++	++	-	-	-	-	+	+	+	+
BASEMENT MEMBRANE	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
PROXIMAL MUSCLE	-	-	+	++	-	-	++	+++	N	-	-	+	-	+	++	+++
HYOID MUSCLE	-	-	+	++	-	-	+	++	N	-	-	+	-	+	++	+++
TRIGEMINAL MUSCLE	-	-	-	+	-	-	-	++	N	N	-	+	-	-	+	+
PERICHONDRAL CAP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MESENCHYME IN GENERAL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] Alcian Blue--pH 1.14

[b] Alcian Blue--pH 2.47

KEY:

N negative activity
- Trace or general background activity
+ Moderate activity
++ Strong activity
+++ Intense activity
++++ Very intense activity

(Table 7).

F. Carbonhydrate staining intensity increased progressively (Table 8). Connective tissue within the muscle stained positively with alcian blue at pH 1.14 and at pH 2.24, while the muscle fibers were PAS+. Muscle fibers also demonstrated some toluidine blue metachromisia.

Muscle development in experimental embryos followed the same histochemical pattern as in control mandibles, even though muscle bundles were smaller and developing fibers sometimes irregularly aligned.

Cartilage development. A. Alkaline phosphatase (Plate VII, 7b and 7e) was found both cytoplasmically and extracellularly within cartilage and perichondrium. Activity was strong prior to chondrification and moderate during the period of chondrification (Table 7). Perichondral activity remained for a longer time than phosphatase activity in the cartilage matrix.

B. Acid phosphatase was confined to the cytoplasm of the small cartilage cells (Plate VII, 7c and 7d). Activity became stronger (Table 7) and was found both cytoplasmically and extracellularly.

C. ATP dephosphohydrolase was also confined to the cytoplasm of cartilage cells (Plate IX, 9e and 9f). As development proceeded, activity became strong intracellularly and intercellularly in the perichondrium (Table 7).

D. AMP dephosphydrolase activity appeared in trace to moderate quantities in the perichondrium and matrix of the cartilage in stage 30 to 33 embryos (7 and 8 days) (Plates IX, 9g and X, 10b). By stage 35 activity was negative in the cartilage and trace activity was present in the perichondrium (Plate XI, 11e).

E. RNA was located cytoplasmically in cartilage cells and in the perichondrium during the entire interval of development observed (Plates IX, 9d and XI, 11a, 11b and 11f).

F. Intensity of the carbohydrate tests demonstrated that chondrification occurred first in the proximal end of Meckel's cartilage and moved distally. Carbohydrates were uniformly distributed by the end of the 7th day of incubation (stages 29-30) and were located cytoplasmically and extracellularly. Alcian blue at pH 1.14 did not stain nuclei (Plate XII, 12d and 12f), while alcian blue at pH 2.47 stained nuclei (Plate XII, 12c and 12e). Alcian blue (Plate XIII, 13a and 13b) and PAS+ (Plate XIII, 13e) staining intensities increased as growth proceeded (Table 8).

Presence of chondroitin sulfate in the cartilage was indicated by toluidine blue metachromasia (Table 8). Hyaluronidase removed this metachromasia (Plates XII, 12a and 12b; XIII, 13c and 13d). Tests for keratosulfate were negative; it was not detected in even the oldest control

mandibles tested (stage 35, 9 days).

No differences were observed in the histochemistry of the cartilage in experimental mandibles. Photomicrographs of acid phosphatase activity (Plate VII, 7f) and ATP and AMP dephosphohydrolase activity (Plate X, 10a and 10b) illustrate normal cartilage development in irradiated mandibles.

Perichondral cap. A. Alkaline phosphatase activity (Plate VIII, 8b) was moderate in the perichondral cap, while the surrounding mesenchyme had moderate to strong activity.

B. Acid phosphatase activity (Plate VII, 7c and VIII, 8a) was much stronger in the cap than the adjacent mesenchyme.

C. ATP dephosphohydrolase activity (Plate IX, 9e) was moderate to strong and was also stronger in the cap than in the adjacent mesenchyme.

D. AMP dephosphohydrolase activity was negative at all times.

E. RNA activity was greater in the cytoplasm of mesenchymal cap cells during the 6th and 7th days of development than in those of the lateral perichondrium (Table 7).

F. All carbohydrate stains exhibited only trace quantities in the cap (Table 8).

Similar patterns of activity occurred in the perichondral caps of experimental embryos.

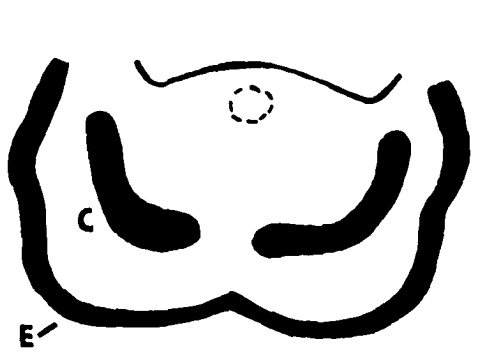
Mesenchymal development. A. Strong alkaline phosphatase activity appeared in the lateral and medial mesenchyme prior to the actual appearance of the membrane bones. As soon as the supra-angular bone (stage 30, 7 days) appeared, activity declined in the surrounding mesenchyme. Activity followed a similar pattern with the formation of the angular, dentary and opercular bones. By stage 35 (9 days) alkaline phosphatase activity was mainly confined to developing membrane bones, i.e., see Figures 27 through 29 for distribution of alkaline phosphatase activity.

B. Acid phosphatase was strong to moderate in the proximal-lateral and distal-lateral mesenchyme, but was present as general background activity in the medial mesenchyme (Table 7).

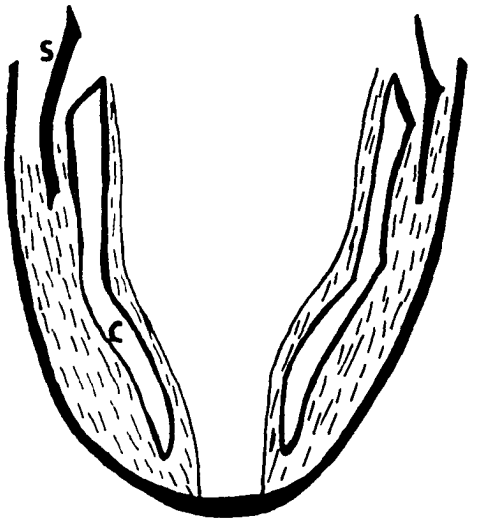
C. ATP dephosphohydrolase activity was moderate in the distal-lateral and medial mesenchyme. Strong activity was present in the proximal mesenchyme, where the greatest developmental activity occurred (Table 7). Proximal muscle, quadrate and proximal Meckel's cartilage (earliest chondrification) and the angular and supra-angular bone (earliest bone development) occurred in this region.

D. Two days after the first AMP dephosphohydrolase activity occurred (stages 26-27, 5 days) in the proximal mesenchyme (Table 7) (Plate IX, 9b), the supra-angular

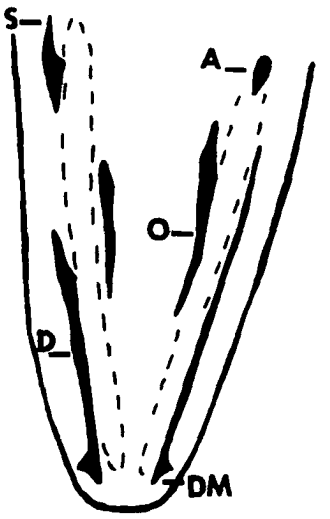
ALKALINE PHOSPHATASE ACTIVITY
(Shaded Areas Denote Activity)



27.



28.



29.

Figure 27. Stage 27 mandible (5 days). C--Meckel's cartilage primordia, E--ectoderm.

Figure 28. Stage 31 mandible (7 days). C--Meckel's cartilage, S--supra-angular bone.

Figure 29. Stage 35 mandible.(9 days). A-- angular bone, D--dentary bone, DM--mentomandibular-gonial bone, O--opercular bone, S--supra-angular bone.

bone appeared with strong activity (Plate X, 10d). Proximal mesenchymal activity declined at this time. Activity extended the entire length of the mandible in the lateral mesenchyme of stage 32-33 (8 days) mandibles before the appearance of the dentary and mentomandibular-gonial bones; see Figures 30 through 32 for the distribution of AMP dephosphohydrolase activity. Strong activity in the distal-lateral mesenchyme preceded dentary-mentomandibular-gonial bone formation.

E. RNA was distributed fairly uniformly throughout the mesenchyme, with activity somewhat greater in the proximal mesenchyme.

In experimental mandibles acid phosphatase activity was greater than in controls, in the lateral and distal mesenchyme. Active site locations depended upon the pattern of cell death found in the mandible, and correlated with histological observations of cell deaths. Acid phosphatase was very intense in mandibles with severe distal and distal lateral necrosis (Plate VII, 7f).

In experimental embryos, alkaline phosphatase and AMP dephosphohydrolase activities in the distal mesenchyme conformed to blunted mandibular contours. Enzymatic intensities did not appear different than in control mandibles. No alterations were apparent in the ATP dephosphohydrolase and RNA activities or in carbohydrate staining intensities.

Bone development. The developmental pattern was

AMP DEPHOSPHOHYDROLASE ACTIVITY
(Shaded Areas Denote Activity)

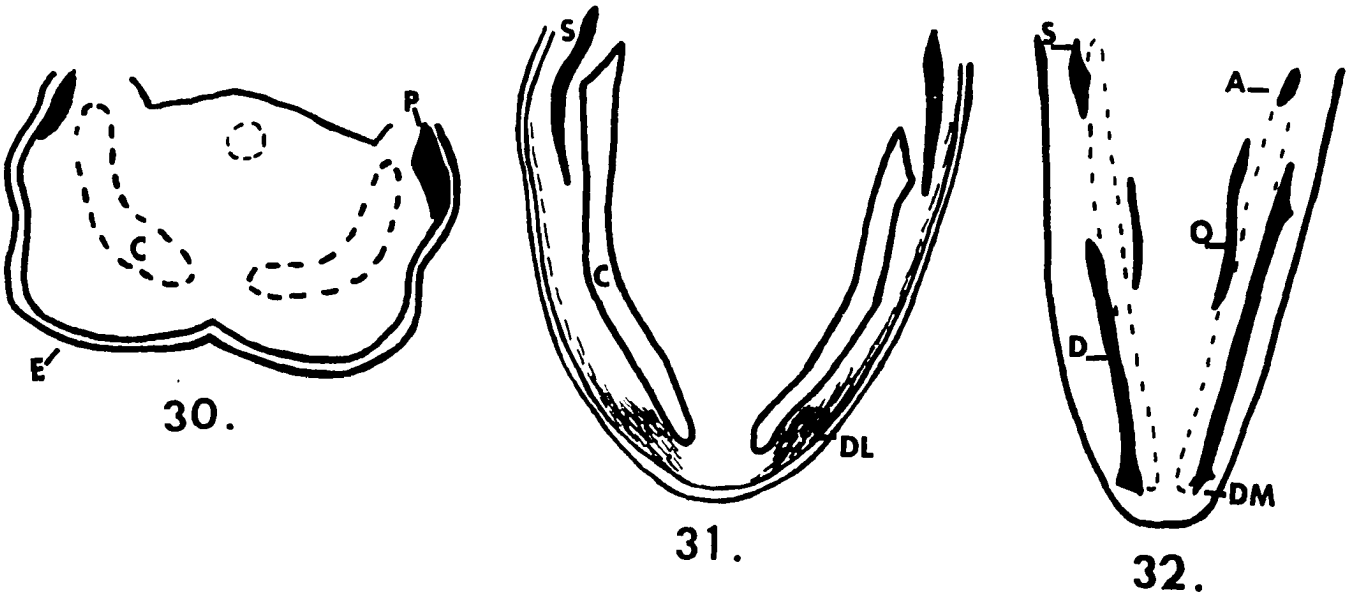


Figure 30. Stage 27 mandible (5 days). C--Meckel's cartilage primordia, E--ectoderm, P--proximal mesenchyme.

Figure 31. Stage 33 mandible (7 1/2 - 8 days). C--Meckel's cartilage, DL--distal-lateral mesenchyme, S--supra-angular bone.

Figure 32. Stage 35 mandible (9 days). A--angular bone, D--dentary bone, DM--dentary-mentomandibular-gonial bone, O--opercular bone, S--supra-angular bone.

the same for all 4 membrane bones. Order of bone development (Table 9) was the supra-angular (Table 10), angular, opercular and dentary.

A. Strong alkaline phosphatase activity occurred in the mesenchyme prior to the appearance of all bones (Plate VIII, 8b). All cells of the periosteum, as well as the osteoblasts and osteocytes contained strong to intense alkaline phosphatase activity. Activity was intense in the osteoid (Plate VIII, 8d), but decreased as trabeculae developed (early mineralization) (Table 10). Membrane bone contours were distinguishable in stage 35 (9 days) mandibles (Plate VIII, 8e) due to strong to intense alkaline phosphatase activity.

B. Acid phosphatase activity became strong in bone primordia when they appeared as dense cell condensations (Plate VIII, 8c) and increased as development advanced (Table 10). Activity was strong in newly formed trabeculae and was particularly intense around the edges.

C. ATP dephosphohydrolase activity (Plates X, 10c and XI, 11d) was similar to that of acid phosphatase, however, activity did not become as intense around the edges of trabeculae.

D. AMP dephosphohydrolase activity was very similar to that of alkaline phosphatase (Table 10). Strong activity occurred in early bone formation (Plate X, 10d) and increased in intensity in osteoid (Plate XI, 11e) and

TABLE 9

HISTOCHEMICAL ACTIVITY--BONE DEVELOPMENT

Histochemical Reaction	Supra-Angular Stages				Angular Stages				Opercular Stages				Dentary Stages			
	30	31	33	35	30	31	33	35	30	31	33	35	30	31	33	35
ALKALINE PHOSPHATASE	+	++	+++	++	-	+	+++	++	-	-	++	++	-	-	+	+++
ACID PHOSPHATASE	+	++	++	+++	-	+	++	+++	-	-	++	+++	-	-	+	++
ATP DEPHOS.	+	++	++	+++	+	+	++	+++	-	-	+	+++	-	-	+	++
AMP DEPHOS.	+	++	+++	++	-	+	++	++	-	-	+	++	-	-	+	+++
RNA	N	-	+	++	N	-	+	++	N	N	-	+	N	N	-	+
ALCIAN BLUE pH 1.14	-	-	-	+	-	-	-	+	N	N	N	-	N	N	N	-
pH 2.47	-	-	+	++	-	-	-	++	N	N	-	+	N	N	N	+
PAS+	-	+	++	+++	-	-	-	++	-	-	-	+	-	-	-	-

KEY:
- N Negative activity
- - Trace activity
- + Moderate activity
- ++ Strong activity
- +++ Intense activity
- ++++ Very intense activity

TABLE 10

HISTOCHEMICAL ACTIVITY--SUPRA-ANGULAR BONE DEVELOPMENT

Location	Acid P.	Alk. P.	RNA	ATP De.	AMP De.	PAS+	Alcian Blue (pH 1.14)	Alcian Blue (pH 2.47)	TEM[a]
MESENCHYME (BEFORE OSTEOID)	+	++	+	+	++	-	-	-	-
PREOSTOE-BLASTS	+	++	+	+	++	-	-	-	-
OSTEOBLASTS	+	+++	++	+	+++	-	-	-	-
OSTEOCYTES	+	+++	++	+	+++	-	-	-	-
OSTEOID	++	++++	+	++	+++	+	+	++	-
PERIOSTEUM	++	++	+	++	++	-	-	-	-
MINERALIZED TRABECULAE	++	+++	-	++	+++	++	+	++	+
TRABECULAE EDGES	+++	++	+++	+	++	++	+	++	+

[a]Toluidine blue metachromasia

KEY:
- \- Trace activity
- \+ Moderate activity
- ++ Strong activity
- +++ Intense activity
- ++++ Very intense activity

newly mineralized bone.

E. RNA became strong in the cytoplasm of the osteoblasts and osteocytes during osteoid formation (Plate XI, 11f) and mineralization (Table 10). Osteoblasts along trabeculae edges contained intense RNA activity.

F. Intensity of carbohydrate staining increased as trabeculae developed and mineralization occurred (Table 10) (Plate XIII, 13a, 13b, 13c, 13d, and 13e). There was some evidence of toluidine blue metachromasia in mineralized trabeculae of the supra-angular bone (most advanced bone development).

In experimental mandibles, ossification appeared to be normal, as indicated by histochemical observations. Bone development was retarded 12 to 24 hr. and thus, the initial enzymatic activities were later in making their appearance. Enzymatic intensities were normal when they occurred. While the blastema of the dentary and opercular bones were present in several embryos sacrificed at an estimated stage 35- (9 days), ossification had not begun. The development of the trabeculae in the angular and supra-angular bones was not as extensive as in control mandibles. Bones were generally thick (Plate X, 10e and 10f) and failed to elongate properly. Each bone molded to the contours (Plate VIII, 8f) of abnormal cartilage and to blunted distal mandibular contours (Plate VIII, 8g). In some

embryos with severe cartilage bends, the dentary-mento-mandibular-gonial bone primordia were either not separate in the mid-line (Plate VIII, 8g) or were in close juxta-position. These bones were normally separate in the mid-line at 9 days of incubation (Plate VIII, 8e).

PLATE VII

Acid and Alkaline Phosphatase Activity (A)

7a Normal embryo--stage 24. Acid phosphatase activity is strong in the ectoderm, on either side of the mid-line and in the numerous necrotic cells and macrophages (arrow) found in the mid-region. (x 49)

7b Normal embryo--stage 27+. Alkaline phosphatase is present throughout the ectoderm, cartilage and muscle primordia, and nerve fibers. C--Meckel's cartilage, N--nerve, T--trigeminal "musculature." (x 49)

7c Control embryo--stage 28+--sacrificed 8 hours after experimental embryos were x-irradiated. Acid phosphatase activity is shown. The nerve fibers and ectoderm are active sites. A number of necrotic cells can be discerned just medial to the distal cartilage tips and along the lateral surface of the cartilage. C--Meckel's cartilage, N--nerve, P--perichondral cap, T--trigeminal "musculature." (x 49)

7d Same as 7c. (Detail--insert) The nerve fibers are shown passing near the proximal end of the cartilage. Necrotic cells (arrows) are found on both sides of the cartilage. C--Meckel's cartilage, N--nerve. (x 105)

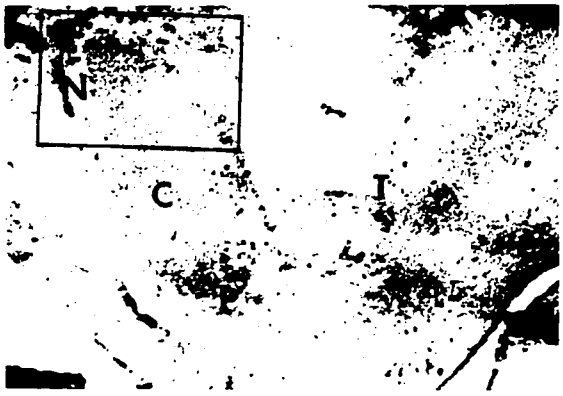
7e Same as 7c. Alkaline phosphatase activity is more intense in the perichondrium than within the cartilage. There is considerable activity in the ectoderm, N--nerve, P--perichondrium. (x 49)

7f Experimental embryo--estimated stage 29--sacrificed 2 days after x-irradiation. Meckel's cartilage is stunted. Considerable acid phosphatase and necrotic cells are present distally (arrows). C--Meckel's cartilage. (x 49)

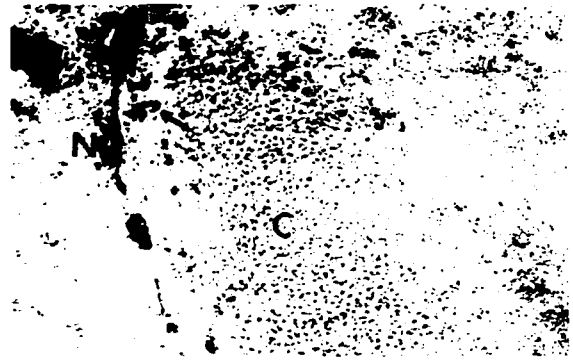
PLATE VII

7 a

7 b



7 c



7 d

7 e

7 f

PLATE VIII

Acid and Alkaline Phosphatase Activity (B)

8a Control embryo--stage 31--sacrificed 2 days after experimental embryos were x-irradiated. Acid phosphatase is intense in the perichondrial cap. Trigeminal muscle primordia and nerve fibers have strong activities. C--Meckel's cartilage, N--nerve, P--perichondrial cap, T--trigeminal "muscle."

8b Same as 8a (adjacent section). Alkaline phosphatase distribution is shown. There is some activity in the perichondrium. Lateral mesenchyme has considerable activity, particularly where the dentary bone will develop. C--Meckel's cartilage, D--dentary primordium. (x 49)

8c Control embryo--stage 31--sacrificed 2 days after experimental embryos were x-irradiated. The proximal end of Meckel's cartilage is shown. Acid phosphatase is intense in the perichondrium and in the supra-angular bone primordium. C--Meckel's cartilage, S--supra-angular bone. (x 49)

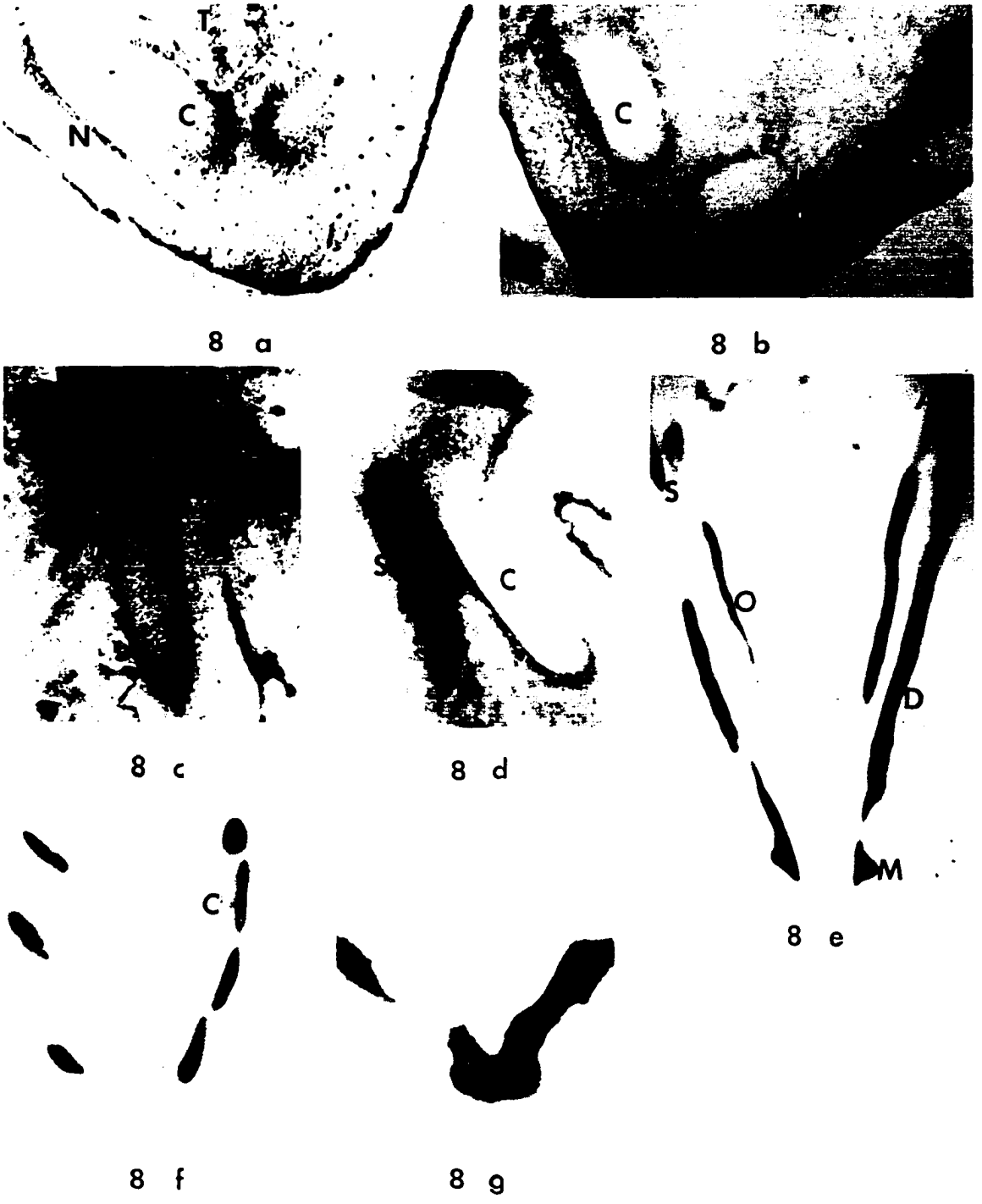
8d Same as 8c (adjacent section). Alkaline phosphatase is moderately strong in the perichondrium and in the supra-angular bone. C--Meckel's cartilage, S--supra-angular bone (x 49).

8e Control embryo--stage 35--sacrificed 4 days after experimental embryos were x-irradiated. Alkaline phosphatase is mainly confined to the membrane bones. The bones parallel Meckel's cartilage, the outline of which cannot be made out. A--angular, D--dentary, M--mentomandibular, O--opercular, and S--supra-angular bones. (x 10)

8f Experimental embryo--estimated stage 34--sacrificed 4 days after x-irradiation. Alkaline phosphatase activity is shown in the abnormally curved membrane bones. The bones have developed to conform with the cartilage curvature. A small section of outlined cartilage is visible. C--Meckel's cartilage. (x 10)

8g Same as 8f. The dentary-mentomandibular bones have fused in the mid-line. (x 49)

PLATE VIII



8 a 8 b

8 c 8 d 8 e

8 f 8 g

PLATE IX

RNA, ATP and AMP Dephosphohydrolase Activity (A)

9a Normal embryo--stage 18. ATP dephosphohydrolase activity occurs in two rod-like areas in the mandibular arches (nerve and presumptive muscle). Acid and alkaline phosphatase activity are localized in the same areas at this stage. (x 49)

9b Control embryo--stage 27--sacrificed 30 minutes after experimental embryos were x-irradiated. AMP dephosphohydrolase activity is localized in the proximal mesenchyme (arrow). The ectoderm contains some activity. (x 49)

9c Control embryo--stage 28--sacrificed 2 hours after experimental embryos were x-irradiated. ATP dephosphohydrolase activity is shown. Active sites are the trigeminal and hyoid "musculature" and the ectoderm. C--Meckel's cartilage, H--hyoid "musculature," T--trigeminal "musculature." (x 49)

9d Same as 9c. RNA is strong in the cytoplasm of cartilage cells and in the ectoderm. C--Meckel's cartilage. (x 49)

9e Control embryo--stage 29+--sacrificed 1 day after experimental embryos were x-irradiated. ATP dephosphohydrolase activity is very strong in the perichondral cap. C--Meckel's cartilage, P--perichondral cap. (x 59)

9f Control embryo--stage 31--sacrificed 2 days after experimental embryos were x-irradiated. ATP dephosphohydrolase activity is shown in the "musculature" to the left of the proximal end of Meckel's cartilage. Intense activity is present in the quadrato-jugal bone. A slight suggestion of the supra-angular bone is present. C--Meckel's cartilage, P--proximal "musculature," QJ--quadrato-jugal bone, S--supra-angular bone. (x 49)

9g Same as 9e (adjacent section). AMP dephosphohydrolase activity is present in the quadrato-jugal bone. A little activity is visible in the cartilage. C--Meckel's cartilage, P--proximal "musculature," QJ--quadrato-jugal bone, S--supra-angular bone. (x 49)

9 a

9 b

9 c

9 d

9 e

9 f

9 g

PLATE X

AMP and ATP Dephosphohydrolase Activity (B)

10a Experimental embryo--estimated stage 32+--sacrificed 3 days after x-irradiation. Cartilage band is shown. ATP dephosphosphohydrolase activity is strong in the perichondrium and trigeminal "musculature." The latter ends where the cartilage bend forms. Activity is also present in the ectoderm and nerve fibers. C--Meckel's cartilage, E--ectoderm, N--nerve, T--trigeminal "musculature." (x 49)

10b Same as 10a (adjacent section). AMP dephosphohydrolase activity is very intense along the distal-lateral sides of the mandible. This activity conforms with the blunted distal end of the mandible. The perichondrium of the bent cartilage is barely outlined (arrow). C--Meckel's cartilage. (x 49)

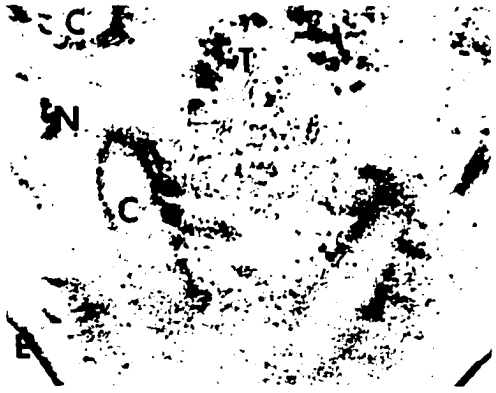
10c Control embryo--stage 30--sacrificed 2 days after experimental embryos were x-irradiated. ATP dephosphohydrolase activity is very intense in the quadrato-jugal and supra-angular bones. Moderate activity occurs in the perichondrium. C--Meckel's cartilage, QJ--quadrato-jugal bone, S--supra-angular bone. (x 49)

10d Same as 10c. AMP dephosphohydrolase activity is confined to the supra-angular and quadrato-jugal bones. QJ--quadrato-jugal bone, S--supra-angular bone. (x 49)

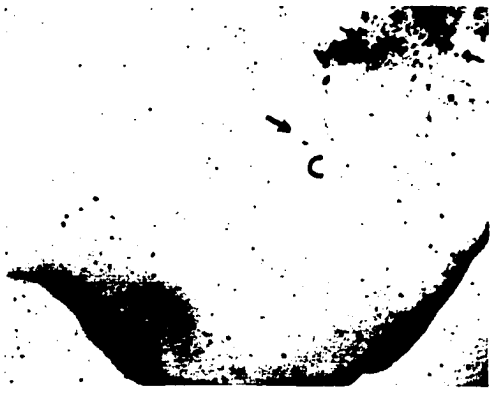
10e Experimental embryo--estimated stage 34--sacrificed 4 days after x-irradiation. ATP dephosphohydrolase activity is shown in the proximal musculature, quadrato-jugal and supra-angular bones. Developing bones are short and thick. Trabeculae are not well developed. C--Meckel's cartilage, P--proximal "musculature," QJ--quadrato-jugal bone, S--supra-angular bone. (x 49)

10f Same as 10e (adjacent section). AMP dephosphohydrolase is confined to the developing bones. Some activity is present in the proximal mesenchyme. M--proximal mesenchyme, QJ--quadrato-jugal bone, S--supra-angular bone. (x 49)

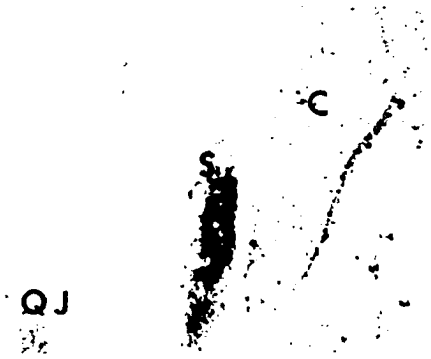
PLATE X



10 a

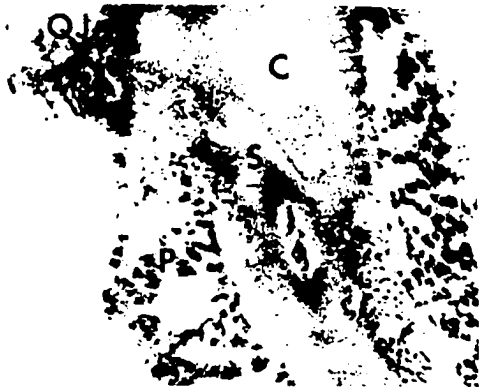


10 b

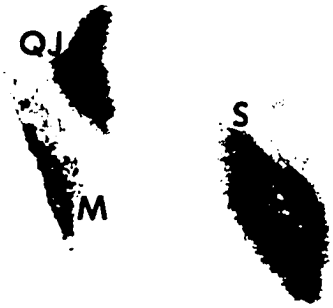


10 c

10 d



10 e



10 f

PLATE XI

RNA, ATP and AMP Dephosphohydrolase Activity

11a Control embryo--stage 30--sacrificed 2 days after experimental embryos were x-irradiated. RNA activity is shown in the quadrato-jugal and supra-angular bone primordia and in Meckel's cartilage. C--Meckel's cartilage, QJ--quadrato-jugal bone, S--supra-angular bone. (x 49)

11b Same as 11a (adjacent section). This section was treated with RNA. Bone primordia can no longer be distinguished. The cytoplasm of the cartilage cells appears as clear areas within the cartilage. The remaining cartilage stain is due to the staining of the cartilage matrix with methyl green-pyronin. C--Meckel's cartilage. (x 49)

11c Control embryo--stage 30--sacrificed 2 days after experimental embryos were x-irradiated. ATP dephosphohydrolase activity is shown in the developing proximal "musculature," the quadrato-jugal bone, the quadrate and a small portion of Meckel's cartilage. C--Meckel's cartilage, P--proximal "musculature," Q--quadrate, QJ--quadrato-jugal bone. (x 49)

11d Control embryo--stage 35--sacrificed 4 days after experimental embryos were x-irradiated. ATP dephosphohydrolase activity is shown in a section of the dentary and opercular bones. C--Meckel's cartilage, D--dentary bone, E--ectoderm, O--opercular bone, T--trigeminal "musculature." (x 49)

11e Same as 11d (adjacent section). AMP dephosphohydrolase activity is mainly confined to the dentary and opercular bones. C--Meckel's cartilage, D--dentary bone, E--ectoderm, O--opercular bone, T--trigeminal "musculature." (x 49)

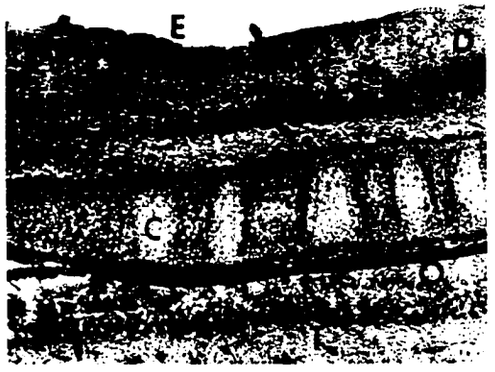
11f Same as 11d (adjacent section). Some RNA is present in the dentary and opercular bones. RNA in Meckel's cartilage is obscured by the staining of the cartilage matrix. C--Meckel's cartilage, D--dentary bone, E--ectoderm, O--opercular bone, T--trigeminal "musculature." (x 49)

PLATE XI

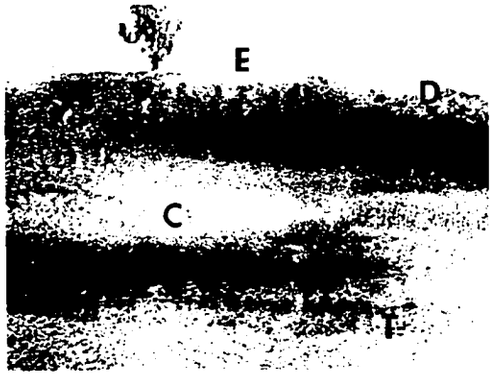
11 a

11 b

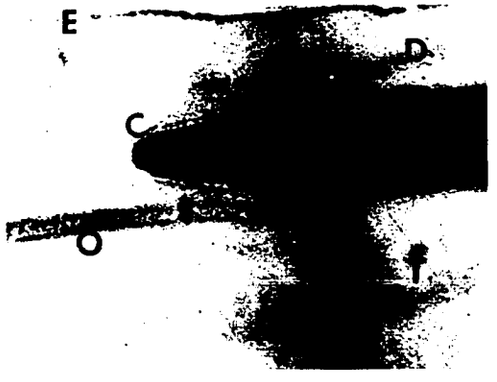
11 c



11 d



11 e



11 f

PLATE XII

Carbohydrate Activity (A)

12a Control embryo--stage 28+--sacrificed 12 hours after experimental embryos were x-irradiated. Toluidine Blue metachromasia is shown in Meckel's cartilage. (x 49)

12b Same as 12a (adjacent section). Metachromatic stain is removed following hyaluronidase treatment. (x 49)

12c Control embryo--stage 29--sacrificed 1 day after experimental embryos were x-irradiated. Alcian Blue (pH 2.47) stains the cartilage matrix and ectoderm. Nuclei are stained. (x 49)

12d Same as 12c (adjacent section). Alcian Blue (pH 1.14) stains the cartilage matrix and the ectoderm. (x 49)

12e Control embryo--stage 27+--sacrificed 4 hours after experimental embryos were x-irradiated. Early staining of the cartilage primordium is shown (Alcian Blue--pH 2.47). The nuclei are stained, but the mandibular nerve is negative. C--Meckel's cartilage, N--nerve. (x 185)

12f Same as 12e. Alcian Blue (pH 1.14) stains the developing cartilage matrix. C--Meckel's cartilage. (x 185)

PLATE XII

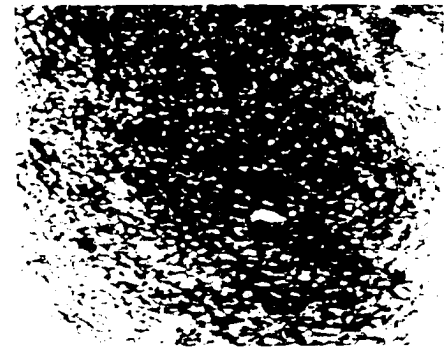
12 a

12 b

12 c

12 d

12 e



12 f

PLATE XIII

Carbohydrate Activity (B)

13a Control embryo--stage 35+--sacrificed 4 days after experimental embryos were x-irradiated. Alcian Blue (pH 1.14) stains the cartilage intensely. The supra-angular bone is slightly stained. C--Meckel's cartilage, S--supra-angular bone. (x 49)

13b Same as 13a (adjacent section). Alcian Blue (pH 2.47) stains the supra-angular bone and Meckel's cartilage. The stain is stronger in the perichondrium and the outer portion of the cartilage than in the interior. C--Meckel's cartilage, S--supra-angular bone. (x 49)

13c Same as 13a (adjacent section). Toluidine Blue metachromasia is strong in the cartilage and perichondrium. C--Meckel's cartilage, S--supra-angular bone. (x 49)

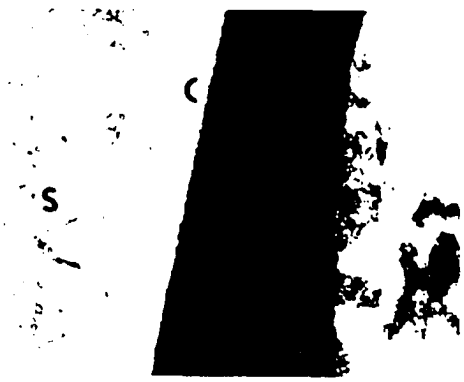
13d Same as 13a. Toluidine Blue staining following hyaluronidase treatment indicates that much of the metachromatic staining in the cartilage is due to chondroitin sulfate. There is little change in the bone stain. C--Meckel's cartilage, S--supra-angular bone. (x 49)

13e Same as 13a. The perichondrium and developing bone is strongly PAS positive. The cartilage matrix is moderately PAS positive. C--Meckel's cartilage, S--supra-angular bone. (x 49)

13f Control embryo--stage 28+--sacrificed 12 hours after experimental embryos were x-irradiated. Alcian Blue (pH 1.14) stains the basement membrane of the ectoderm. B--basement membrane, E--ectoderm, M--mesenchyme. (x 371)

13g Experimental embryo--estimated stage 29--sacrificed 12 hours after x-irradiation. Alcian Blue (pH 1.14) is shown in the basement membrane. B--basement membrane, E--ectoderm, M--mesenchyme. (x 371)

PLATE XIII



13 a

13 b

13 c

13 d

13 e

13 f

13 g

CHAPTER IV

DISCUSSION

Sensitive Period

Results of this study indicate that the embryonic chick mandible is most sensitive to the effects of x-irradiation at 5 days of incubation or approximately stage 27. A dose of 625r permits maximum survival of 5-day embryos with the production of slight to extreme forms of the anomaly, without massive hemorrhaging. Since the same dose produces only slight mandibular reductions in 4 and 6 day embryos, it is believed that the range of the sensitive period is well delineated, i.e., between the extremes of 4 and 6 days. Higher doses applied at the 4 and 6 day extremes result in more severe reduction, but not with the frequency or severity of those occurring at 5 days at lower doses. Results are consistent with the 5 day irradiation sensitivity of the mandible mentioned by Goff (unpublished data).

Boric acid, pilocarpine (Landauer, 1952, 1953), azaserine (Dagg & Karnofsky, 1955) and eserine sulphate (Agarwal, 1956) had their greatest effect on mandibular development when administered at 4 days of incubation. The difference in stimulus-response time may reflect the time required for

absorption and accumulation of an adequate concentration of the teratogen into the mandible, or to its mode of action, or to a combination of these factors. It is entirely possible that chemical agents and x-irradiation mediate their effects at different points and in different manners. Landauer (1952) found that boric acid effects were almost completely eliminated by the addition of riboflavin. The effects of azaserine were not diminished by the addition of riboflavin (Dagg & Karnofsky, 1955). It seems reasonable that azaserine and boric acid either effect different metabolic pathways or different points in the same pathway as suggested by Dagg and Karnofsky. It is likely that chemicals which cause mandibles to be reduced, but not bent, have a greater effect on the cartilage, while a chemical causing cartilage curvature (eserine sulphate) has a greater effect on surrounding tissues.

Anomalous Mandibular Development

Goff (1959) noted hemorrhages in the mandibles of x-irradiated chick embryos, drawing attention to the possibility that hemorrhages are causal factors in the production of anomalous mandibular development. Embryos dying acutely, after receiving high (750r) irradiation doses, also have hemorrhages in the brain, eye, limbs, and back. It is to be expected that such hemorrhaging generally results in death. Histological observation of a sampling of embryos x-irradiated at a dose of 750r demonstrate massive hemorrhage

in the majority of mandibles. Such blood vessel rupture and flooding of developing tissues obstructs the ability to analyze specific primordial sensitivity. However, mandibles of embryos receiving 625r rarely develop severe hemorrhages, but anomalous development results, including the least and most severe forms. Therefore, hemorrhage as a causal factor in the production of the mandibular anomaly in this study is discounted.

Mandibular blisters can not be correlated with anomalous mandibular development. Since a number of control embryos exhibited small clear blisters beneath their mandibles, it is apparent that the posterior ectoderm-mesenchymal connection of the 5 and 6 day embryonic mandible is very loose, which tends to allow for the accumulation of fluid in an embryo exposed to a teratogenic agent inducing edema as one of its effects. In embryos where severe hemorrhage occurs posterior to Meckel's cartilage, fairly normal development proceeds. It may be that mandibular hemorrhages drain into clear blisters leaving relatively unaltered mandibular primordia. When mesenchymal necrosis is very severe, anomalous development results, with or without the presence of a blister or hematoma. Grabowski (1964), in the study of the etiology of hypoxia-induced malformations in the chick embryo, observed occasional hematomas underneath the chin without any appreciable effect on mandibular development.

Radiation sensitivity correlates with the amount of cell death in developing mandibular tissues. Judging from the anomalies that occur, the cells of the mesenchyme that are most affected are the preconnective tissue cells, which lie in the lateral mesenchyme. Within the proximal portion of the mesenchyme are migrating bone primordial cells (Jacobson & Fell, 1941) which are apparently more resistant, since membrane bones were not specifically affected. Presumptive connective tissue cells make up the bulk of the remaining lateral mesenchyme, with the exception of the dentary bone primordia, which also appears resistant. Bone (quadrate), cartilage and muscle are also present as mesenchymal condensations at the sensitive period, but subsequent development shows them to be less sensitive than the lateral connective tissue. It is only in the more severe anomalies that muscle, bone and cartilage are significantly affected. The variation in sensitivity of connective tissue primordia and that of bone, cartilage and muscle condensations is responsible for the variation in the manifestation of mandibular anomalies discussed in the following sections.

Cell Death Pattern I

When the connective tissue primordia are very lightly affected, the result is the development of smaller, but normally formed mandibles. In such mandibles Meckel's cartilage, muscle and bone primordia are only lightly necrotic. Histogenesis of these tissues occurs normally as indicated

by histological and histochemical observations.

Cell Death Pattern II

When the connective tissue primordia are affected to a greater degree and cartilage, muscle and bone primordia are not proportionately more severely damaged, cartilage bending results. Mean lengths of Meckel's cartilage in mandibles with slight reduction (Cell death pattern I) and in mandibles with cartilage bends are very similar. These results indicate that Meckel's cartilage elongates in spite of the reduction of more sensitive mandibular tissues. Histological and histochemical evidence supports the earlier work of Jacobson and Fell (1941) in showing that the perichondral cap is the main growing point of Meckel's cartilage. Even though the perichondral cap is not severely affected and distal cartilage growth continues, cell death in the perichondral cap results in the growth of a less sturdy distal portion of Meckel's cartilage. The combined factors of the growth of a weakened distal cartilage, within the confines of a mandible of reduced space, results in the mechanical bending of the distal end. The angle of each cartilage bend is dependent upon the degree of mesenchymal sensitivity. As the destruction of presumptive connective tissue increases cartilage bends become more acute. Folding of the distal end of the cartilage is a consequence of extreme mesenchymal necrosis. Histogenesis of Meckel's cartilage appeared normal as indicated by the histological

and histochemical analysis of this study.

Comparison of control and experimental cartilage contours indicate that the major bend occurs within the distal 1/3 of Meckel's cartilage. This distal portion of Meckel's cartilage is marked by a curvature in control embryos, which may indicate a weak point in the cartilage. The fact that the major bend occurs more proximally in 4-day embryos demonstrates that the stress point is also dependent upon the incubation age. It is to be expected that cartilage bends would form more proximally when connective tissue is damaged at an earlier period in development.

The trigeminal musculature terminates at the initiation or the apex of the major bend. Muscle growth apparently stopped abruptly at this point in some embryos as indicated by the clumping of developing fibers. Muscle is hindered in its proper elongation, perhaps due to reduction in mandibular space, or its growth may require normal cartilage elongation. Since muscle primordia are more sensitive than the cartilage, reduction in muscle cells (as a result of cell death) may also be in part responsible for the termination of distal muscle growth. The histochemistry can only be interpreted as normal even though muscle arrangement and size relationships are altered.

Bone development (initial ossification) is retarded from 12 hr to 1 day, which is consistent with the overall retardation of the embryo. Trabeculae formations are not

as advanced as in controls of the same age of incubation. The contours of the membrane bones conform with the abnormally curved or bent cartilage. In addition, membrane bones are short and blunted in their long dimensions, which may be a consequence of the anomalous development of Meckel's cartilage. Jacobson and Fell (1941) suggested that normal cartilage elongation may be necessary for the proper stretching and growth of the membrane bones, and this idea is supported by these observations. Otherwise histogenesis, as revealed by histological and histochemical analysis, is normal.

The fusion, or near fusion, of the dentary and mentomandibular-gonial bones in the mid-line of the mandible in a number of x-irradiated embryos may be the consequence of both the reduction in distal-lateral connective tissue and the bending of Meckel's cartilage. Both factors can act to bring the bone primordia closer together in the mid-line. Erdmann (1940) indicated that the mentomandibular-gonial bones fuse in the mid-line in 10 day embryos, which is later in development than the observations of this study.

The tongue conforms with the shape of the mouth cavity in normal development (Hamilton, 1965). Histological observations reveal that the tongue contour in mandibles of cell death pattern II is altered, but conforms with the shape of the lower jaw. Observations in this study do not

reveal bulging or excessive growth. The tongue as a factor in the production of the anomaly is eliminated.

Cell Death Pattern III

When dense mesenchymal blastemas are severely affected, along with connective tissue primordia, stub mandibles result. The perichondral cap, trigeminal muscle and dentary and mento-mandibular-gonial bone primordia are heavily damaged. With the main growing point of Meckel's cartilage destroyed, along with the bulk of the distal tissue condensations, severely shortened mandibles are expected results.

Intense acid phosphatase activity accompanies the severe necrosis in this cell death pattern. The occurrence of massive necrosis in the distal end of the mandible 2 days after x-irradiation in some embryos, suggest that secondary cell deaths occurred, since necrotic cells are generally fairly well removed in the majority of embryos by 2 days after x-irradiation. Another possibility is that necrosis is so severe initially that macrophages are incapable of removing all of the dead cells by this time.

Results are similar, in some respects, to those reported by Skalko (1965) in his study of the effect of Co^{60} radiation on development and DNA synthesis in the rat embryo. DNA synthesis was reduced, as compared to controls, by both 135r and 270r, in the 1st 12 hr post radiation, but greater reduction in DNA synthesis occurred following 270r, the teratogenic dose. However, by 24 hr after radiation DNA synthesis

rates were the same in the controls and both sets of experimental embryos. Skalko hypothesized that embryos receiving 135r were capable of apparently complete recovery, while the damage was so great initially in embryos receiving 270r that complete recovery was not possible, despite the fact that a greater increase in DNA synthesis had occurred in these embryos.

It is apparent in this study that the variety of anomalies resulting from teratogenic doses is due, in part, to differences in sensitivity between embryos and their ability to recover from the radiation damage. Malformations in cell death pattern II result due to the differential sensitivity of the mandibular tissues. Mechanical bending of the cartilage is a result of the differential sensitivity of presumptive connective tissue and cartilage. In cell death pattern III radiation damage is initially so severe in bone, muscle and cartilage primordia that compensation is not possible.

Hicks (1954) in analyzing radiation damage in embryonic mice and rats suggested that malformations represented a balance between damage and the capacity of any given anlage or developing zone to repair.

Argyris, (1968) in a review on the growth of the tissues of the skin induced by damage of various kinds, indicated that the stimulation of growth of each of the tissues is probably specific, i.e. requires damage of the

tissue itself for the induction of its proliferation. The greater DNA synthesis observed by Skalko (1965) in rat embryos following radiation at a teratogenic dose may be the result of a similar phenomenon in embryonic tissues. DNA synthesis studies on chick embryos following a wide range of doses of x-irradiation may shed light on this question.

While maxilla reduction is slight in comparison to that of the mandible, the tendency for a significant increase in reduction at 6 days of incubation indicates greater sensitivity at this time. The maxilla develops later than the mandible during embryonic development, thus accounting for its greater sensitivity at 6 days.

Cornification occurs in mandibles of experimental embryos allowed to develop beyond the usual time of sacrifice, i.e., 15, 16 days of incubation. Since mandibles exhibiting all extremes of the anomaly develop cornified mandibles, it is apparent that the cornification of the epidermis is not prevented by x-irradiation.

Normal Cell Death

The role of cell death in normal morphogenesis of the chick embryo is incompletely known. Basic to this report is the resolution of normally occurring cell death in the developing mandible. Local cell deaths occur during embryonic processes such as folding, evagination, invagination, part separation, fusion, Lumen formation, ossification

and the ingrowth of tissue (Glücksmann, 1950). Necrosis observed in the mid-line of 3 and 4 day mandibles in this study verifies the results of Jacobson and Fell (1941). They concluded that the necrotic cells provided room for ingrowing myogenic cells. Necrotic cells present in the mesenchyme distal-medial to Meckel's cartilage up to stage 30 (7 days) may provide space for the distal elongation of Meckel's cartilage. This seems likely since muscle primordia are located medial to the cartilage primordia from 4 days on during development. Strong acid phosphatase activity in this region correlates with the cell deaths in this area. It is possible that these necrotic cells are the lingering result of the more massive cell death occurring at 3 and 4 days, but their specific location and persistence in the area makes this seem unlikely.

Necrotic cells observed in the proximal osteogenic center are in agreement with the earlier results of Jacobson and Fell (1941). They indicated that the dead cells may divide the osteogenic center into 3 parts, thus separating the supra-angular, angular and opercular primordia.

Cell deaths in developing spinal ganglia of the chicken embryo have been well documented (review, Glucksmann, 1950). As presumptive neuroblasts and spongioblasts die, cells that would become Schwann cells for the ensheathment of fibers of such cells, may degenerate because there is no stimulation for their differentiation. This may be

responsible for some of the cell deaths observed along the mandibular nerve fibers in this study.

Normal Histogenesis

Bone

Initial ossification occurs at 7 days for the supra-angular, 7 1/2 to 8 days for the angular, 8 days for the opercular and 8 to 9 days for the dentary. The ossification sequence in this study follows, fairly closely, the time scale of Erdmann (1940). The proximal-distal appearance of membrane bones correlates with the distal migration of primordia determined by Jacobson and Fell (1941).

The activities of alkaline phosphatase and RNA observed in this study are in agreement with activity patterns for bone reported in a review by Cabrini (1961). Carbohydrate staining results agree with those reported for bone in a review by Pritchard (1956).

Strong ATP dephosphohydrolase activity in osteoblasts, osteocytes and developing trabeculae indicate that this enzymatic activity is involved in the formation of membrane bone. Bourne (review, 1956) stated that ATP was a component of preosseous cartilage. It was indicated that ATP may or may not provide phosphate used in the formation of bone salt, but may be concerned in the production of energy for the breakdown of glycogen. It seems likely that the high levels of ATP dephosphohydrolase in osteoblasts

and osteoid of the membrane bones of this study demonstrate dephosphorylation of ATP is important to the mineralization of membrane bones.

Results of this study suggest that AMP dephosphohydrolase activity may be correlated with some morphogenetic process in the proximal and distal-lateral osteogenic centers. The first appearance of (stage 26) activity occurs in the proximal mesenchyme, adjacent to the ectoderm, and suggests an ectodermal-mesenchymal interaction (e.g., tertiary induction) prior to the formation of the proximal portions of the membrane bones. As development proceeds AMP dephosphohydrolase activity becomes widespread in the proximal mesenchyme and as the quadrato-jugal bone of the upper jaw forms, followed by the supra-angular, angular and opercular bones in turn, activity becomes very intense within each primordium. AMP dephosphohydrolase activity may be related to a specific morphogenetic property of the mandible, i.e., induction of membrane bone formation.

As development continues, AMP dephosphohydrolase activity proceeds distally (following the first occurrence) in the mesenchyme adjacent to the ectoderm. A continuing ectoderm-mesenchymal interaction may accompany the distal migration of the primordia. The appearance of strong activity in the distal-lateral mesenchyme prior to the intense activity that appears shortly thereafter in the mentomandibular-gonial and dentary bones may indicate an ectoderm-mesenchymal

interaction similar to that suggested proximally. Very intense levels of AMP dephosphohydrolase activity in osteoid, mineralized bone, osteoblasts and osteocytes indicates its continuous involvement well along in the bone formation process.

It would be worthwhile observing the AMP dephosphohydrolase activity in the developing membrane bones of the upper beak as well as in the remainder of the skull. Results of this study suggest that a similar wave of activity is at least involved in the formation of the quadrato-jugal bone. If additional study still indicates AMP dephosphohydrolase involvement in membrane bone formation, it would be valuable to continue this investigation employing other methods such as tissue exchange, transplantation, and tissue culture.

Cartilage

Cell condensations (perichondral cap) noted at the distal tips of Meckel's cartilage are growth centers for cartilage elongation (Jacobson & Fell, 1941). The activity of acid phosphatase, RNA and ATP dephosphohydrolase verify the distal growth center. Cytoplasmic basophilia and the intensity of carbohydrate staining indicates a proximal-distal pattern of chondrification, which was also the conclusion of Jacobson & Fell (1941).

The distribution of acid mucopolysaccharides in the cartilage matrix was similar to that previously described for the chick embryo (Rolle, 1967 and Hall, 1968). The failure

of Meckel's cartilage to stain positively for kerato-sulphate is in agreement with Muir (1964). Muir indicated the presence of kerato-sulphate after the 15th day in the cartilage of the chick embryo.

The appearance of alkaline phosphatase in early cartilage formation is not in complete agreement with the results Milaire (1963) obtained in studying the mouse limb bud. Milaire noted considerable ATP and AMP dephosphohydrolase, as well as acid phosphatase in the developing perichondrium of, but not in, preosseous cartilage. These enzymatic activities are observed in the perichondrium and cartilage proper in this study. The fact that Meckel's cartilage grows interstitially as well as appositionally and does not calcify may account for its difference in enzymatic distribution. AMP dephosphohydrolase activity was only barely present in Meckel's cartilage during chondrification.

It has been reported (review, Cabrini, 1961) that RNA is localized in nearly all cartilage cells, which was the case in this study. Cabrini pointed out that it was only after calcification (which was not observed in Meckel's cartilage) had occurred that RNA activity diminished.

Muscle

No description of the embryological development of lower jaw musculature in the chicken embryo is available. The transverse trigeminal muscle may correspond to the mylohydoideus muscle of the duck, Anas platyryhnchos (Kallius,

1905), which runs to a tendon in the mid-line of the lower jaw. In the duck, the definitive position for the muscle is no further forward (distal) than the mid-point of the mandible. It is evident from this study that the transverse trigeminal muscle in the chicken will also have a subterminal location.

The longitudinal trigeminal muscle may correspond to the geniohyoid muscle of the duck, also described by Kallius, which runs medial-lateral to Meckel's cartilage and attaches near the apex of the lower jaw.

In a review by Beckett and Bourne (1960) it was pointed out that work carried out on the histochemical development of embryonic muscle was almost nil. The histochemistry of adult avian muscle has been studied in considerable detail (review, George & Berger, 1966). Enzymatic localizations are described for particular structures within adult muscle fibers (sarcotubules, mitochondria, myofibrils). While study of cellular detail was not attempted in the present work, it is shown that acid phosphatase and ATP dephosphohydrolase activity are present in developing fibers. The former was described in a review (Beckett and Bourne, 1960) as being present in chick muscle only in early stages of differentiation from the mesoderm. Such results are not indicated in this study.

ATP and ATP dephosphohydrolase are universally present in biological systems. The intense ATP dephosphohydrolase activity found in developing trigeminal and hyoid muscle fibers may be correlated with developing functional ability of the

musculature. AMP dephosphohydrolase activity is not outstanding in early muscle development, but it has been demonstrated in adult pigeon pectoralis muscle fibers (review, George & Berger, 1966).

In this same review, glycogen was mentioned as a component of avian skeletal muscle. No conclusive statements may be made from this study concerning the presence of glycogen, but it may be that some of the strong PAS+ staining is attributable to glycogen. Observations of this study indicate that alkaline phosphatase is present in early forming muscle masses, but not appreciably in later development (fiber formation). These results are consistent with those reviewed by Beckett & Bourne (1960) to the effect that alkaline phosphatase had been noted in the fibrils of myotomes of the 5 mm human embryo, but not in later fetuses.

The connective tissue staining of the developing skeletal muscle indicates the formation of sulphated and carboxylated mucopolysaccharides (Lev & Spicer, 1964).

Comparison with Congenital Anomalies

Most congenital mandibular anomalies are not adequately described in the Literature to permit an interpretation on the basis of observations in this study. The descriptions of the following anomalies at least allow general speculation.

In the congenital anomaly in which the lower beak is a mere vestige (Marble, Harpers & Hammers, 1944) it seems reasonable to assume that the growing point for Meckel's

cartilage is disrupted. It may be speculated that the distal mesenchyme, including proximal bone and muscle condensations are also affected.

The failure of connective tissues to develop properly may be the primary factor involved in the production of the congenitally reduced mandibles described by McGibbon (1946). The fact that extreme forms of the anomaly resulted in the bending of the cartilage may indicate a more severe connective tissue reduction and that a gradation is involved in the genetic effect. It can be assumed with considerable assurance that the congenital effects are in operation during the 4th and particularly the 5th day of incubation in the production of both of these malformations.

General Comments

While the histochemical tests employed in this study, with the exception of acid phosphatase, did not reveal significant metabolic alterations in early histogenesis, it is realized that such an effect is not ruled out. The fact that the products of the breakdown of induced necrotic cells are added to the environment of developing tissues indicates a change in the chemical composition of this environment. That cell death is primarily involved in the production of the mandibular anomaly is not questioned, but a positive statement can not be made indicating that biochemical changes are not involved.

CHAPTER V

SUMMARY AND CONCLUSIONS

1. The sensitive period for the induction of reduced mandibles in chick embryos, with x-irradiation, was determined to be 5 days of incubation or stage 27.

2. Blister formation could not be correlated with mandibular reduction.

3. A possible correlation between nerve growth and cell death of the surrounding tissue is suggested. Cell deaths around the distal ends of Meckel's cartilage through the 7th day may aid distal cartilage elongation. Necrosis in the mid-line of 3 day mandibles and in the proximal osteogenic centers were in agreement with cell death sites determined by Jacobson and Fell (1941).

4. Variations in mandibular anomalies can be correlated with the incidence and frequency of cell deaths. It is apparent in this study, that the variety of anomalies resulting from teratogenic doses is due, in part, to differences in sensitivity between embryos and their ability to recover from the radiation damage.

5. Smaller, but normally proportioned mandibles developed as a result of a weak response to irradiation. Connective

tissue was lightly affected and the overall mandibular response was uniformly distributed.

6. Mandibular cartilage bends representing an intermediate response, resulted from differentially sensitive connective tissue primordia. The development of the less sensitive primordia of bone, cartilage and muscle was modified by the insufficiency of connective tissue.

7. The primordia of all the tissues were severely affected in a disto-proximal order in the most severe form of the anomaly. Extremely shortened mandibles resulted.

8. The overall growth of the x-irradiated embryos was retarded. Estimated stages for experimental embryos averaged 12 hr to 1 day behind those of the controls.

9. Cartilage formation (or chondrification) was not altered in experimental embryos as indicated by the histochemical tests.

10. Bone development in experimental embryos (initial ossification) was retarded from 12 hr to 1 day.

11. The bones of the experimental embryos conformed to the abnormally curved and or bent cartilage. Membrane bones were short and blunted in their long dimensions. Histochemistry was normal.

12. AMP dephosphohydrolase mesenchymal activity was correlated with the activity of the osteogenic centers in preparation for bone development; an induction mechanism may be involved.

LITERATURE CITED

Agarwal, I. P. (1956) Morphogenetic effects of eserine sulphate. J. Anim. Morph. Physiol. 3, 63-74.

Argyris, T. S. (1968) Growth induced by damage. Advances in Morphogenesis, 7, 1-43.

Barka, T. & Anderson, J. (1963) Histochemistry, Theory, Practice and Bibliography, pp. 92-105. New York, Hoeber.

Beckett, E. B. & Bourne, G. H. (1960) Histochemistry of developing skeletal and cardiac muscle. Structure and Function of Muscle, pp. 87-109, New York, Academic Press.

Bellairs, R. (1961) Cell death in chick embryos as studied by electron microscopy. J. Anat. 95, 54-63.

Bensley, S. H. (1934) On the presence, properties and distribution of the intercellular ground substance of loose connective tissue. Anat. Rec. 60, 93-109.

Bourne, G. H. (1956) Phosphatase and bone. The Biochemistry and Physiology of Bone, pp. 251-286, New York, Academic Press.

Cabrini, R. L. (1961) Histochemistry of ossification. Intern. Rev. Cytol. 11, 283-306.

Clapp, N. K. (1964) LD_{50} (24 hrs) Observations in the chick embryo after exposure to x-rays. Radiation Res. 22, 457-462.

Dagg, C. P. & D. Karnofsky. (1955) Teratogenic effects of azaserine on the chick embryo. J. Exp. Zool. 130, 555-572.

Erdmann, K. (1940). Zur zntwicklungsgeschichte der knocken im schadel des huhnes bis zum zeitpunkt des ausschlüpfens aus dem ei. Z. Morph. Ökol Tiere. 36, 315-400.

George, J. C. & Berger, A. J. (1966) Avian Myology. pp. 25-114, New York, Academic Press.

Glücksman, A. (1950) Cell deaths in normal vertebrate ontogeny. Biol. Revs, Cambridge Phil. Soc. 26, 59-86.

Goff, R. A. (1959) The acute lethal response of the chick embryo to x-radiation. J. Exp. Zool. 141, 477-497.

Gomori, G. (1941) Distribution of acid phosphatase in the tissues under normal and under pathologic conditions. Arch. Pathol. 32, 189-199.

Grabowski, O. T. (1964) The etiology of hypoxia-induced malformations in the chick embryo. J. Exp. Zool. 157, 307-326.

Gray, P. (1952) Handbook of Basic Microtechnique, p. 29, New York, Blakiston Company.

Grüneberg, H. (1961) Genetical studies on the skeleton of the mouse 27. The development of oligosyndactylism. Genet. Res. 2, 33-42.

Hall, B. K. (1968) Histochemical aspects of the differentiation of adventitious cartilage on the membrane bones of the embryonic chick. Histochemie. 16, 206-219.

Hamburger, V. & Hamilton, H. L. (1951) A series of normal stages in the development of the chick embryo. J. Morph. 88, 49-92.

Hamilton, H. L. (1965) Lillie's Development of the Chick, pp. 376-377, New York, Holt, Rinehart and Winston.

Hicks, S. P. (1952) Some effects of ionizing radiation and metabolic inhibition on the developing mammalian nervous system. J. Pediat. 40, 489-513.

Hicks, S. P. (1954) Mechanism of radiation anencephaly, anophthalmia, and pituitary anomalies. Arch. Pathol. 57, 363-378.

Hinchliffe, J. R. & Ede, D. A. (1967) Limb development in the polydactylous talpid mutant of the fowl. J. Embryol. Exp. Morph. 17, 385-404.

Jacobson, W. & Fell, H. B. (1941) The developmental mechanics and potencies of the undifferentiated mesenchyme of the mandible. Quart. J. Micro. Sci. 82, 563-591.

Kallius, E. (1905) Beiträge zur entwickelung der zunae. II Teil. Vögel (Anas bachas L., Passer domesticus L.) Anat. Hefte. Abt. 1. 28, 307-586. (As Cit. Romanoff, 1960).

Karnofsky, D. A., Patterson, P. A. & Ridgeway, L. P. (1950) The toxicity of roentgen rays to the chick embryo. Am. J. Roent. 64, 280-288.

Kingsbury, J. W., Allen, V. G. & Rotheram, B. A. (1953) The histological structure of the beak in the chick. Anat. Rec. 116, 95-115.

Kurnick, N. B. (1955) The quantitative estimation of desoxyribose nucleic acid based on methyl green staining. Exp. Cell Res. 1, 151-159.

Kury, G. & Craig, J. M. (1967) The effect of mitomycin on developing chicken embryos. J. Embryol. Exp. Morph. 17, 229-237.

Lamoreux, W. F. (1942) Hereditary chondrodystrophy in the fowl. J. Heredity. 33, 275-283.

Landauer, W. (1952) Malformations of chicken embryos produced by boric acid and the probable role of riboflavin in their origin. J. Exp. Zool. 120, 469-508.

Landauer, W. (1953) On teratogenic effects of pilocarpine in chick development. J. Exp. Zool. 122, 469-483.

Lev, R. & Spicer, S. (1964) Specific staining of sulfate groups with alcian blue at low pH. J. Histochem. Cytochem. 12, 309.

Lillie, R. D. (1948) Histopathologic Technique. pp. 25-28. Philadelphia, Blakiston Company.

Marble, D. R., Harper, J. A. & Hammers, E. V. (1944) Inheritance in the domestic fowl of a lethal condition affecting both mandibles. Poultry Sci. 23, 406.

McGibbon, W. H. (1946) The inheritance of short lower mandibles in the fowl. Poultry Sci. 25, 406.

Milaire, J. (1962) Detection histochimique de modifications des ebauches dans les membres en formation chez la Souris oligosyndactyle. Bull. Acad. Roy. Med. Belg., Cl. Sc., s.V. 48, 505-528.

Milaire, J. (1963) Etude morphologique et cytochimique de developpement des membres chez la Souris et chez la Taupe. Arch. Biol. 74, 129-317.

Mowry, R. W. (1963) The special value of methods that color both acidic and vicinal hydroxyl groups in the histochemical study of mucins. With revised directions for the colloidal iron stain, the use of alcian blue G8X and their combinations with the periodic acid-Schiff reaction. Ann. N. Y. Acad. Sci. 106, 402-423.

Mulnard, J. (1955) Contribution a la connaissance des enzymes dans l'ontogenese. Les phosphomonoesterases acide et alcoline dans le developpement du Rat et la Souris. Arch. Biol. 66, 525-685.

Muir, H. (1964) Chemistry and metabolism of connective tissue glycosaminoglycans (mucopolysaccharides). Int. Rev. Conn. Res. 2, 101-154.

Nelsen, O. E. (1945) The use of certain phthalate compounds in embryology. Stain Tech. 20, 129-130.

Opie, E. L. & Lavin, G. I. (1946) Localization of ribonucleic acid in the cytoplasm of liver cells. J. Exp. Med. 84, 107.

Pritchard, J. J. (1956) The osteoblast. The Biochemistry and Physiology of Bone, pp. 179-212, New York, Academic Press.

Robinson, R. L. & Bacsich, P. (1958) Improving the staining qualities of toluidine blue. Stain Tech. 33, 171-173.

Rolle, G. K. (1967) Histochemical study of ribonucleoproteins and mucopolysaccharides in developing bone of normal and tetracyline-treated chick embryos. Anat. Rec. 158, 417-431.

Romanoff, A. L. (1960) The Avian Embryo, Structural and Functional Development, pp. 455-459, New York, Macmillan Co.

Saunders, J. W. & Gasseling, M. T. (1962) Cellular death in morphogenesis of the avian wing. Devel. Biol. 5, 147-178.

Scott, J. E., Dorling, J. & Quintarelli, G. (1964) Differential staining of acid glycosamineglycans by alcian blue in salt solutions. Biochem. J. 91, 4-5.

Skalko, R. G. (1965) The effect of Co^{60}-radiation on development and DNA synthesis in the 11-day rat embryo. J. Exp. Zool. 160, 171-182.

Stearner, S. P., Sanderson, E. J. & Christian & Tyler, S. A. (1960) Modes of radiation death in the chick embryo. I. Acute syndromes. Rad. Res. 12, 286-300.

Wachstein, M. & Meisel, E. (1957) Histochemistry of hepatic phosphatases at a physiologic pH. Am. J. Clin. Path. 27, 13-23.

Whitten, J. A. (1968) Cell death during early morphogenesis: parallels between insect limb and vertebrate limb development. Science 163, 28-29.

Yasui, K. & Hayashi, Y. (1967) Morphogenesis of the beak of the chick embryo: Histological, histochemical and autoradiographic studies. Embryologia, 10, 42-74.

APPENDIX

SUMMARY TABLES OF DATA

TABLE A

DATA ON NUMBER AND INCUBATION CONDITIONS OF EGGS

Exp. No.	Date	No. Eggs.	Stage X-Rayed Range	Storage Hours	Storage Temp °C	Incubation Ave. Temp. °F	Incubation Ave. Rel. Hum. %
Ia	5/6/68	56	24-27	3	24	100.0±0.5	51±3.0
Ib		56	21-25+	15	24		
Ic		56	22-24+	28	24		
IIa	5/24	68	28-29-	15	24	100.2±0.7	55±1.0
IIb		68	27-28	27	24		
IIc		68	26+-27	34	24		
IIIa	6/12	68	26-27	15	24	100.1±0.4	54±5.0
IIIb		68	26-27	15	24		
IIIc		68	26-27	15	24		
IVa	6/14	66	26-27	8	20	100.4±0.4	56±7.0
				9	24		
IVb		66	26-27	8	20		
				9	24		
IVc		66	26-27	8	20		
				9	24		
V	6/27	108	29-29+	16	24	100.0±0.5	51±3.0

TABLE A (CONTINUED)

Exp. No.	Date	No. Eggs	Stage X-Rayed Range	Storage		Incubation	
				Hours	Temp. °C	Ave. Temp. °F	Ave. Rel. Hum. %
VI	7/20/68	48	20+-24+	16	24	100.0±0.5	54±4.0
VII	12/9/69	48	23-24+	36	18	100.2±0.3	50±2.0
				13	24		
VIII	7/11/68	204	26-27	16	24	100.3±0.2	54±1.0
IX	7/20	175	26-27	16	24	100.0±0.5	54±1.0
X	8/5/69	54	27	19	10	100.1±0.2	54±2.0
				8	24		
		48	27	19	10		
				20	24		
XI	8/13	52	24-27	5	20	100.0±0.5	54±3.0
		52	26-27-	37	10		
				13	24		
XII	8/13	72	25-26	--	--	99.9±0.2	50±1.0
XIII	12/9	72	27-28-	11	18		
				12	24	100.2±0.3	50±2.0

TABLE B

DATA ON FERTILITY, VIABILITY AND SURVIVING EMBRYOS (%)

Exp. No.	Infertile	Inviable Before Exp.	Dead Controls (End)[a]	Living Controls (End)[a]	Dead X-Rayed (End)[a]	Living X-Rayed (End)[a]	Staged Controls	Accident	Total No. Eggs
I	3.57	2.37	0.59	12.5	35.1	25.6	19.04	1.18	168
II	2.94	0.98	1.47	16.1	41.1	20.6	16.6	0.00	204
III	0.98	0.98	1.47	22.06	22.06	40.69	11.76	0.00	204
IV	1.50	3.03	1.01	17.17	32.83	35.35	9.09	0.00	198
V	1.96	1.47	1.85	9.26	15.74	28.70	11.11	26.85	108
VI	0.87	1.31	0.00	22.9	6.25	43.7	27.1	0.00	48
VII	10.4	6.25	0.00	20.7	6.2	39.3	16.5	0.00	48
VIII	3.92	4.41	0.49	39.20	0.49	43.62	7.84	0.00	204
IX	0.87	1.31	1.14	42.8	15.4	29.7	10.28	0.57	175
X	3.92	1.96	1.96	25.48	9.80	45.08	11.76	0.00	102
XI	5.77	2.88	0.96	22.15	3.84	51.84	11.50	0.96	104
XII	2.78	0.00	0.00	25.0	4.16	54.18	13.9	0.00	72
XIII	11.1	4.2	0.00	19.4	8.8	45.8	11.1	0.00	72

[a](End) = end of experiment

TABLE C

HISTOLOGICAL AND HISTOCHEMICAL STUDY

Exp. No.	Dose (r)	Stage X-Rayed	Staged Prior to Exp.	No. Embryos			No. Embryos		
				X-Rayed	Survived	%	Controls	Survived	%
VIII	625	27	16	90	89	98.8	81	80	98.7
IX	750	27	18	79	52	65.8	77	75	97.4
Xa	625	27	6	32	28	87.5	16	16	100.0
Xb	625	27+	6	24	18	75.0	12	10	83.3
XIa	625	26+	6	31	29	93.5	12	12	100.0
XIb	625	26+	6	27	25	92.6	12	11	91.7
XII	625	26	10	39	36	92.3	18	18	100.0
XIII	650	27+	8	39	33	84.6	14	14	100.0

TABLE D

SERIAL SACRIFICE DATA FOR EXPERIMENTS VIII AND IX

Time Post X-Rayed	No. Embryos		Stages	
	X-Rayed	Controls	X-Rayed (Estimated)	Controls
30 MIN.	4	3	27, 27+, (2)28	(2)27, 27+
2 HOURS	3	2	27-, 27, 27+	27, 28
4 HOURS	4	2	27-, 27, (2)28-	27, 27+
8 HOURS	7	2	(2)27+, 28-, (3)28, 28+	27-, 28-
12 HOURS	8	2	27+, (2)28-, (5)28	28, 28+
1 DAY	6	2	(2)28+, (4)29-	(2)29
2 DAYS	3	2	(2)30-, 31-	31, 32-
3 DAYS	3	2	31, (2)32	33, 34
4 DAYS	3	2	(3)35	(2)35+

Mandibles were embedded in paraffin and sectioned through the length of the mandible at 10 microns. All embryos were stained in Harris' Hematoxylin and Eosin.

TABLE E

SERIAL SACRIFICE DATA FOR EXPERIMENT VIII

Time Post X-Rayed	No. Embryos		Stages	
	X-Rayed	Controls	X-Rayed (Estimated)	Controls
4 HOURS	3	2	26+, 27, 28-	27, 27+
8 HOURS	3	2	28-, (2)28	27, 28-
12 HOURS	2	1	(2)28	28
1 DAY	2	2	(2)28+	(2)29
2 DAYS	2	2	(2)30-	(2)31

Mandibles were embedded in paraffin and sectioned sagitally at 10 microns. Sections were stained in Harris' Hematoxylin and Eosin.

TABLE F

SERIAL SACRIFICE DATA FOR EXPERIMENT X

Time Post X-Rayed	No. Embryos		Stages	
	X-Rayed	Controls	X-Rayed (Estimated)	Controls
30 MIN.	4	2	(2)27-, 27+, 28+	(2)27
2 HOURS	4	1	26+, (3)27-	27
4 HOURS	4	2	(2)26, 27, 28	(2)28
8 HOURS	4	2	(2)28, (2)29	(2)28-
12 HOURS	4	1	(3)28, 29-	28
1 DAY	4	1	(3)28+, 29-	29-
2 DAYS	4	1	28, 29-, (2)29	31-
3 DAYS	2		(2)33	
3.5 DAYS	1	1	34-	34
4 DAYS	2	1	34, 35	35

Mandibles were frozen and sectioned with a Cryostat. Acid and alkaline phosphatase reactions were run on alternate sections of each embryo.

TABLE G

SERIAL SACRIFICE DATA FOR EXPERIMENT XI

Time Post X-Rayed	No. Embryos		Stages	
	X-Rayed	Controls	X-Rayed (Estimated)	Controls
30 MIN.	5	2	27, 27-, (3)27	(2)27-
2 HOURS	4	2	26, (2)27-, 28	26, 27
4 HOURS	4	2	26, 26+, (2)27	26, 27+
8 HOURS	5	2	27, 27+, 28-, (2)28	(2)28-
12 HOURS	5	2	(5)28	28, 28+
1 DAY	4	2	28, (2)28+, 29-	(2)29+
2 DAYS	4	2	29, (2)30-, 30	30, 31
3 DAYS	4	2	32, (2)32+, 33	33+, 34-
4 DAYS	4	2	34-, 34+, (2)35-	35, 35+

Embryos were frozen and sectioned with a Cryostat. Four reactions were run (alternate sections) on each embryo. ATP and AMP dephosphohydrolase, DNA-RNA and DNA were tested for their localizations.

TABLE H

SERIAL SACRIFICE DATA FOR EXPERIMENTS XII AND XIII

Time Post X-Rayed	No. Embryos		Stages	
	X-Rayed	Controls	X-Rayed (Estimated)	Controls
3-6 HOURS	11	5	27-, 27, (2)27+, 28	26+, 27-, 27, 27+, 28
12 HOURS	6	4	(2)28+, (4)29-	(4)28+
1 DAY	5	2	28+, (3)29-, 29	(2)29
2 DAYS	3	1	30, 31-, 31	31+
3 DAYS	3	3	32-, 32, 33	33+, 34-, 34
4 DAYS	4	3	33+, 35-, 35	(2)35, 35+

Mandibles were frozen and sectioned with a Cryostat. The following reactions were run (alternate sections) on each embryo: PAS; Toluidine blue, Hyaluronidase treatment followed by Toluidine blue; Alcian blue in an acetate buffer (pH 2.47) and Alcian blue in an acetate buffer (pH 1.14). Several embryos in the youngest group and in the oldest group were treated with Alcian blue in an acetate buffer (pH 5.7) containing .4M $MgCl_2$ and .9M $MgCl_2$.