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AGE DETERMINATION STUDIES IN MARINE TURTLES

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## Abbreviations:

AFIP = Armed Forces Institute of Pathology,  
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MNH = U.S. Museum of Natural History, Smithsonian Institution,  
Washington, D.C.

NZP = National Zoological Park, Smithsonian Institution,  
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## DISCLAIMER

The opinions in this paper do not necessarily reflect the opinion of the Armed Forces Institute of Pathology or of the Department of Army.

## PREFACE

Reliable age determination techniques are indispensable for developing sound demographic studies which are in turn essential for a thorough understanding of the population dynamics and management problems of animal populations. Sea Turtles, despite the fact that they have been studied intensively for three decades, have virtually evaded the demographer. There is no documented way to determine the age of a turtle, other than by interpreting from tag returns. Hence, there are virtually no empirical demographic data apart from those which are easily accessible from nesting beaches.

Curiously, there is an immense body of literature on age determination - especially involving wildlife and fisheries managers and their respective game and fish species. Many reliable techniques have been available for decades, but even the more obvious analyses have never been applied to any species of turtle, marine or otherwise. We are at the proverbial "square 1".

In addition, it is important to realize that the specialized techniques required for preparation of hard tissues involve a lengthy period of trial and error to be workable. Four employees of the Smithsonian Institution, working on a related project, and including an experienced, certified histotechnician needed 9 months to develop a useful protocol. Various teams of marine mammal biologists with many years of research and considerable financial support are still grappling with basic inconsistencies in fundamental age determination work.

The present project, to make a beginning with sea turtles, finally began after several reductions in proposed scope and funding, resulting in a project with a principal investigator and an assistant, each working

half time for 12 months with \$ 231 for supplies. Midway through the project institutional costs increased, ending the funding a month early.

Nonetheless, a beginning has been made, and work can now continue with more direction. Several promising avenues for future research have been illuminated and critical, difficult-to-obtain specimens have been acquired and prepared for future work. Despite the humble beginning, the ball is now rolling.

## GENERAL INTRODUCTION

Numerous changes occur in an organism throughout its life. Embryology addresses those changes that occur before birth, and gerontology, those that have occurred at old age. Yet, the steady process of age related changes which every organism experiences often passes without attention. The variety of phenomena involved touches every organ system; for example, the subjects described in a review of gerontology cover most of the biology of Man (see Shock, n. d.).

These age related changes are of interest in their own right, but a better understanding of the phenomena provides the biologist with interpretive tools. Age determination is one of the more important and more obvious of these.

A variety of age related phenomena are discussed in this report, although time and facilities have been available to develop protocols and do experiments with only a few techniques. This extensive, broad reaching approach is important, for this project was designed to investigate and develop techniques in a field where they had never been applied. A great deal more research is needed to compare and refine techniques.

STEPS INVOLVED IN AGE DETERMINATION ANALYSIS

OBTAIN SPECIMEN(S) FOR ANALYSIS,

TRANSPORT SPECIMEN(S) TO LABORATORY FOR ANALYSIS,

GROSS PREPARATION OF SPECIMENS,

MICRO PREPARATION OF SPECIMENS,

CHEMICAL PREPARATION OF SPECIMENS,

ANALYSIS OF PREPARATIONS,

INTERPRETATION OF RESULTS,

REPORTING OF RESULTS.

## COLLECTING

Suitable specimens must be found, and with endangered marine turtles this normally means salvaging animals that are found dead or will be killed by local peoples. Knowing about, and finding, these specimens involves both thorough preparation and luck. However, it is usual that the time and expense involved in this first step is badly underestimated.

Consider in the present context the difficulty in knowing of, and then obtaining, known age specimens: notices were published in various national and international journals, hundreds of letters were written and many hours were spent on the telephone in attempts to locate material. But finding a potential source is only the beginning of a long and frustrating process. For three years we have been attempting to get unique material from Thailand, and have even had assistance from the U.S. Embassy, but still there has been no success. Even closer to home, the very agency funding the project had rare knowledge specimens. In one case these were offered to the project but then destroyed; in another instance other specimens were offered to a totally different project (on dolphins) and then destroyed.

Collecting and salvaging permits will usually be required by the host country where specimens are to be collected, and if U.S. residents are involved in the collecting, U.S. permits will also be needed. To obtain U.S. permits it takes no less than six months - often much more. Foreign permits are highly variable in the difficulty to be obtained: in Cayman they take a few hours, in Egypt they take an hour or so - if one has prepared a "prepermit", in Peru and Ecuador they take about a month, and in Mexico they may take years (if possible at all). Hence, while most of the civilized world has entered the "jet age", the common field

biologist has been frequently cast back into the dark ages with the need to comply with lengthy and complicated permissions.

Depending on the specimen, its condition, and what is needed, certain collecting equipment is required. In the very least plastic bags and jars, good labels, a knife and pencil and notebook must be available. If soft, decomposable tissues are collected they will need to be preserved by freezing or in fixative such as buffered 10 % formaldehyde. The most important point is that the collector be adequately equipped. It is all too common to underestimate the time and financial support required to make USEFUL biological collections.

## TRANSPORTING

Once specimens are collected they must be transported to the laboratory where the analysis will be done. Preparations for transportation depend on the condition and volume of materials to be moved. Wet and frozen materials require more care than do dry specimens. Freight costs must also be carefully considered, especially when bulky and heavy specimens are involved, and even more so when special conditions must be met (eg. with frozen materials).

Permits that were necessary for collecting will need to be displayed on demand, as will export and import permits. These are not always the same. In Mexico, at the time of writing, at least three different ministries claim authority over the exportation of marine turtle specimens; the application for, and granting of, each permit is apparently a totally independent event.

As with collecting, it is common to underestimate the time, effort and expense involved in the successful transporting of scientific specimens.

## SPECIMENS OBTAINED FOR AGE DETERMINATION STUDIES

Critical to age determination work are specimens of known age which are required as standards. During this project, the largest collections of known age specimens of sea turtles in a scientific institution has been assembled, including examples of five species and hundreds of individuals. These are listed in the following table 1. The collection salvaged from Cayman Turtle Farm involves over 100 specimens including bones and delicate eye lenses, and ranges from a few months to 14 years of age.

In addition, numerous contacts were made for other known-age specimens. These are listed in the following table 2.

Before the project began, 100 specimens of Olive Ridley Turtles, Lepidochelys olivacea, were salvaged from the fishery at Oaxaca, Mexico. These include heads and flippers. During the project collections were made of delicate ear and eye bones, which show a potential value for age determination but are rarely represented in museums collections. More than 100 skulls of the olive Ridley and more than 30 skulls of Leatherback turtles, Dermodochelys coriacea, were collected in Ecuador and Peru. These not only represent the largest collections of adult skulls of these species, but also the most turtle ear and eye bone material available in any museum. The preparation, cleaning and cataloguing, of these specimens is a lengthy process and by early 1983 most of this material will be available for age determination studies.

Table 1. Known-age specimens that have been acquired.

Species	Locality	Age (Years)	Number	Material acquired	Source
<u>Chelonia mydas</u>	Cayman Turtle Farm	0.1 to 14.0	130	eye lenses, skulls limb bones, eye and ear bones.	J. Wood
<u>Chelonia mydas</u>	?	2.3	1	entire	W. Friar *
<u>Lepidochelys kempfi</u>	Galveston, Texas	0.1 to 1.0	440	entire	J. Leong
<u>Caretta caretta</u>	Florida	20.0	1	part of skeleton (remainder to be acquired)	R. Witham
<u>Caretta caretta</u>	Florida	3.0	1	entire	R. Witham
<u>Caretta caretta</u>	Zurich, Switzerland	10.0	1	entire	R. Honegger
<u>Caretta caretta</u>	Florida	1.2	1	entire	E. Phillips
<u>Caretta caretta</u>	?	3.3	1	entire	W. Friar *
<u>Eretmochelys imbricata</u>	?	4.0	1	entire	W. Friar *
<u>Dermodochelys coriacea</u>	Florida (Mass.)	0.6	1	entire	E. Phillips

\* On loan from AMNH

Table 2. Contacts that have been developed for known-aged specimens.

Species	Locality	Age (Years)	Number	Material available	Source	Problems
<u>Chelonia mydas</u>	Bonin Islands, Japan	1 - 3	?	Skeleton	Y. Kurata	Permits & shipping
<u>Chelonia mydas</u>	Bonin Islands, Japan	6	1?	Skeleton	Y. Kurata	Permits & shipping
<u>Chelonia mydas</u>	Bonin Islands, Japan	4 or 5	1?	Skeleton	Y. Kurata	Permits & shipping
<u>Chelonia mydas</u>	Phuket, Thailand	8	2	Entire	H. Chansang	Permits
<u>Chelonia mydas</u>	Florida, U.S.A	?	Several	? Entire	Christianson	Permits
<u>Caretta caretta</u>	U.S.A.	8	1	? Entire	J. K. Langamorer	No response
<u>Caretta caretta</u>	Florida, U.S.A	10	1	Skeleton	F. Berry	Destroyed ?
<u>Lepidochelys olivacea</u>	Mexico	3	Several	Entire	Christianson	Permits
<u>Lepidochelys olivacea</u>	Phuket, Thailand	10	8	Entire	H. Chansang	No response
<u>Dermochelys coriacea</u>	Ceylon	2	1	?	Mrs. Ratnapala	No response

## SELECTING THE SPECIMEN

Recording structures normally grow by lamellar accretion, but not all tissues exhibit the same lamellar structure. For example, bone is constantly being remodeled as the older, inner layers are resorbed by osteoclasts, and the mineral salts are recycled into the body's general pool. The resorbed bone is in the center, or medulary area, of the bone and takes on a spongy or trabecular form; it is called by all of these adjectives. The outer, or cortical, bone is compact, and it is this part of the tissue where the lamellar structure, produced by accretion of new bone at the intermembraneous surface, is most conspicuous. The tissue and form of growth layers is destroyed and reorganized by the osteoclasts during reabsorption. Hence, the ideal bone for examination of lamellar, or growth layers has a high ratio of cortical to medulary bone; Smirna (1974) commented on this.

Radiographs provide a rapid means of identifying those bones with the most compact area. In sea turtles the humerus and femur show the highest amount of compact bone, and the anterior end of the coracoid before the epiphysis also shows a relatively high amount of compact bone. Most other bones are highly porous, as shown in both radiograms and cross section preparations. Hence, we recommend using the humerus and femur for cross sectioning.

Bones of the skull and vertebrae centra may show external growth marks (see "Externally Visible Growth Marks", page 81 ). In addition, the columella (ear bone) and sclerotic ossicles have a layered structure and warrant further investigations.

## PREPARING THE SPECIMEN

Generally the hard tissues of recording structures are surrounded by soft tissues like muscle and tendon. The presence of these soft tissues prevents accurate measurements of bones and other structures, which are needed in studies of growth. In addition, once the hard structures are collected from the animal, the organic substances within (e.g., collagen) are subject to decay, so some form of preservation is required. Each specimen needs to be cleaned and preserved.

There are numerous ways to clean bones and other hard tissues, and this is something of an art among museum specialists. Boiling, macerating (i.e., allowing the specimen to rot in water); digesting proteins with trypsin or antiformin, or "bugging" (i.e., feeding the specimen to dermestid beetle colonies so that they eat off all soft tissue) are common cleaning procedures. However, each of these may effect the tissues to be studied in a different way; for example, boiling is strongly discouraged by pathologists at AFIP. However, there seems to have been no study of the comparative effects of different cleaning techniques. This warrants further investigations.

Preservation techniques are equally variable and untested. Tissues may be frozen, air dried, or fixed in a variety of conventional histological preservatives such as buffered formaldehyde (of varying strengths), Bouin's fixative, Zenker's fixative, or alcohol (about 70%). Standard procedure at AFIP involves fixation in about 5% formaldehyde and storage in formalol; however, a comparative study is needed to provide the best preparation technique for age determination work.

## STUDIES OF GROWTH LAYERS IN BONE FOR AGE DETERMINATION:

## With Special Reference To Marine Turtles

Age determination techniques are commonly based on interpretations of growth layers in "recording structures", i.e. hard tissues whose normal growth incorporates a record of periodic phenomena. The structures investigated include: chitinous exoskeletons (Neville, 1963; 1967); crayfish gastroliths (Neville, 1967); calcium carbonate skeletons of scleratinion corals (Dodge and Vaisnys, 1980); mollusk shells (Rhoads and Lutz, 1980); polychaete jaws (Olive, 1982); barnacles (Bourget, 1980); dermal scales (Peabody, 1961); special calcified structures of the middle ear ("otholiths or sagittae" - Panella, 1980), ear wax plugs (Nishiwaki, 1957); keratinous horns (Simpson, 1972), claws (Peabody, 1961); baleen plates (Lockeyer, 1981) and hair (Harris and Kalmus, 1948); tooth replacement and tooth ware (Larson and Taber, 1980), tooth cementum (Klevezal and Kleinenberg, 1967); tooth denteen (Nishiwaki, et al., 1958; Klevezal and Kleinenberg, 1967; Hohn, 1980; Perrin and Myrick, 1980; Lockyer, et al., 1981); and bone (Peabody, 1961; Klevezal and Kleinenberg, 1967; Castanet and Cheylan, 1979; Hemelaar and Van Gelder, 1980). All of these structures are characterized by being harder than "normal" body tissues and by growing by periodic accretion of material.

The value in "recording structures" is that they maintain a record of an animal's growth and are not anabolized or destroyed, but rather continue to accumulate material throughout the life of the animal. As these structures are hard, they are not subject to the normal decomposition experienced by most body tissues. But this hardness also produces special problems in preparations and examination, and special

techniques have to be developed and used.

The purpose of this report is to explain simply, but in rather more detail than found in most articles in scientific journals, the problems we have encountered and the procedures we have used in attempting to produce preparations useful for the study of growth layers. Although some of these techniques have been used for centuries there is considerable debate about both the techniques and the interpretations. The best illustration of these fundamental problems is in the recent symposium and workshop on age determination in cetaceans (Perrin and Myrek, 1981) which brought together most of the world's experts who have been engaged in this work for years or even decades. The results of the workshop proceedings show that virtually each laboratory has its own unique techniques and that there is tremendous variation in interpretation of identical preparations when read by experienced observers.

Some observers exploit this imprecise nature of the technique to "prove" pre-existing hypothesis. Hence, age determination studies are as much an art as a science, and there is an urgent need to provide more detailed and repeatable descriptions of methods and more objective interpretations of results. The object of this report is to stimulate these goals.

It is also relevant that problems in establishing basic demographic studies and managing exploited animal populations are most pressing in the "Third World". Hence the use of sophisticated equipment, materials, and procedures relying on "state of the art" technology from industrialized nations excludes a large body of workers with pressing needs. For this reason we have, whenever possible, tried to use simple, universal explanations and to rely on simple, inexpensive techniques and equipment.

Clearly, this is not the last word in age determination techniques, but it will have accomplished its purpose if it stimulates more objective work not only in the USA but also in less developed countries.

## SECTIONING BONE

A common way to investigate growth layers is by sectioning, or cutting cross sections of the recording structure. The process is much like examining rings in a tree trunk. In marine turtles this involves scale and bone. As discussed above, scale generally reveals a poor record because of sloughing and abbrasion. Hense, bone is the most obvious place to investigate growth layers. As Peabody (1961) showed bone has been sectioned for examination of growth layers in a wide variety of vertebrates:

Fishes (Meunier, et al., 1979);

Amphibians (Hemelaar and Van Gelder, 1980);

Testudines (Mattox, 1935; Castanet and Cheylan, 1979);

Squamate reptiles (Bryuzgin, 1937; Castanet, 1973; 1978; Smirna, 1974);

Dinosaurs (De Ricqles, 1974);

Birds (Van Soest and Van Utrecht, 1971); and

Mammals (Klevezal and Kleinenberg, 1967; Pascal and Castanet, 1978).

Before sectioning, the bone has to be selected, prepared by cleaning and preserving, and the best place in the bone must be chosen. These points are discussed briefly under the appropriate headings above.

In the older literature sectioning is referred to as "grinding (or polishing) thin sections" because this is exactly what was done. We have not used that technique, so the description that follows involves the use of special equipment. However, reference is made to the less expensive older method.

CUTTING THE BONE

Most small bones, less than about 10cm long, can be held directly in a variety of chucks of the Isomet 11-1180 Low Speed Saw (e.g. chucks 11-1183, 11-1184, 11-1185, or 11-1187). Wafers, sections about 100 um (micron) thick, and "pieces", sections more than 1 mm thick, can be cut directly from the bone held in any one of these chucks. We maintain the convention of cutting sections at the proximal end first and moving distally with sequential cuts, thus making a series of sequential parallel cuts.

Larger bones have to be cut into smaller pieces with a saw; a bone saw, hacksaw, jig saw or band saw can be used. Two cuts remove the selected piece; it is important that these cuts be parallel to each other and perpendicular to the long axis of the bone. If a humerus is used, the proximal cut should pass just distal to the deltopectoral crest (Figure Q). The distal cut should be distal to the greatest constriction of the shaft. If a femur is used, both proximal and distal cuts should be either side of the greatest constriction.

Portions of shaft cut from large bones on a band saw can be cut on the Isomet held in any one of a variety of chucks, (including those listed above and 11-1186). The latter is simplest as the distal side of the cut portion can be fixed to the chuck with hot paraffin. This is both fast and simple. Again, we cut sections from the proximal end first.

Cutting on the Isomet is preferable to cutting on a band saw as the thin, diamond studded blade wastes much less of the sample by producing less bone dust. It is also possible to cut wafers as thin as 100 um fairly regularly on the Isomet, although successful use requires a certain amount of experience.

The high concentration 4-inch blade (11-4244) can be used for most cuts (Low concentration blades are recommended for cutting objects harder than bone). The 3-inch blade (11-4243) wastes less bone because it is half as thick (0.15 mm rather than 0.30 mm). However, this blade, produced from pulverized metal, is weak and easily distorts from a planar surface if one edge is loaded.

Water with enough soap or detergent to break the surface tension serves as a lubricant (Alcohol with glycerin was also recommended but not tried by us).

The variable speed control knob of the Isomet is marked from "0" to "10", and normally set at "5". Four weights are provided from the manufacturer: 25, 50, 75 and 150 g, and we normally use the 50 g. The faster and/or heavier the setting, the more rapid the cut will be, but the slower and lighter the setting, less stress is put on the blade and it will more closely approximate a plane, producing a straight even cut.

Pieces and wafers are numbered sequentially from proximal to distal with a soft (No. 1) pencil or India ink on the side with the greatest relative amount of spongy bone (i.e. away from the surface to be cut or examined). The average thickness of each piece and wafer is measured and recorded, and the pieces and wafers are stored. We recommend that pieces be 3 to 4 mm thick and wafers be less than 100  $\mu$ m thick. Pieces can be transferred directly to 10% buffered formalin.

It is recommended by the manufacturer that every other cut be used to "dress" the blade by cutting a thin section of a carborundum stick (11-1190). Because of the inconvenience of replacing chucks, and chances of returning the bone to a slightly different position (thereby wasting a cut and bone by having to trim to get two parallel cuts), we

cut a series of sections before dressing, but always dress between changes of sample.

There is always a temptation for the novice to hand hold the dressing stick or other object, against the blade.

THE MOVING BLADE SHOULD NEVER COME IN CONTACT  
WITH ANYTHING NOT HELD SECURELY IN A CHUCK.

When beginning a cut, start the blade rotating slowly, through the lubricant fluid, and GRADUALLY lower the weighted swivel arm with chuck and sample onto the blade. The speed can be changed during a cut, but weights should be changed by lifting up the swivel arm first.

A valuable source of technical information is Mr. Walter Wardzala, Product Quality Metalographer, Buehler Ltd., 41 Waukegan Road, P.O.Box 1, Lake Bluff, Illinois 60044, USA.

The most inexpensive technique to make thin sections of bone involves the old method of grinding or polishing thick pieces to make them thinner. Frost (1958) describes this in detail. All that is needed is abrasive paper (Carborundum), a perfectly flat surface onto which to place the paper (e.g., a piece of glass 20 cm<sup>2</sup>), and a saw.

### DECALCIFICATION

Hard tissues provide unique information for age determination for they act as recording structures, but they also present special problems for examination. The very structures which store age-related information make it impossible to examine these tissues with standard histologic technique, and the mineral salts, notably calcium carbonate ( $\text{CaCO}_3$ ), must be removed. After decalcification the tissue, supported mainly by collagen fibers, can be sectioned by histological procedures developed for "normal" soft tissues, although with certain important modifications.

A variety of solutions are conventionally used for decalcification, and most of these are acids which reduce the calcium salt and remove it from the tissue. The AFIP (Armed Forces Institute of Pathology) manual lists no less than four such solutions (Luna, 1980), and nearly a dozen techniques are listed in some texts (e.g., Sheehan and Hrapchak, 1980). There are also commercially available "decal" agents whose ingredients are trade secrets. RDO<sup>®</sup> is one of the more common commercial preparations (Wu and Michaels, 1969; Power, 1977; Anon., n.d.). These solutions vary in the speed with which they act, and hence their harshness to the tissues being decalcified. We have found good and repeatable results with a solution which is simple, relatively safe, rapid, cheap and comparatively mild; this solution is preferred by the AFIP.

## HYDROCHLORIC ACID-FORMIC ACID DECALCIFICATION (H-F)

## SOLUTIONS:

A. Hydrochloric Acid-Formic Acid Decalcifier:

Tap water	840 ml
Hydrochloric acid (HCl) (Concentrated, 37%)	80 ml
Formic acid 88% (HCOOH)	80 ml

Pour one acid at a time slowly into the water. Store in a covered plastic or glass container until needed.

## PROCEDURE:

1. Labeled bone pieces should be placed in a volume of the Hydrochloric acid-Formic acid decalcifier (H-F) at least 10 times greater than the volume of bone to be decalcified. We found that 100-150 ml of the H-F solution was sufficient to decalcify one piece of bone. The large volume ensures that the solution will not become saturated with calcium ions, and thereby stop or slow the decalcification process. Each piece can be kept in a plastic tissue cassette (see page 59).
2. If the bone is to be sectioned on a freezing microtome, about 24 hours of decalcification in the H-F solution is sufficient. If the bone is to be paraffin embedded, the piece should be decalcified for about 48 hours. The length of time required for decalcification will depend upon the size and thickness of the piece. Regardless of the total time required for the decalcification, the H-F solution should be replaced with fresh solution every 24 hours.
3. We found that the best indication of decalcification was when the bone was slightly flexible when held and moved in the hand

(wash the piece with tap water before handling and/or use gloves and be careful not to damage the tissue by bending it excessively).

We did not find radiograms helpful for determining the degree of decalcification of the small pieces used here, but other laboratories regularly depend on this technique.

4. Following decalcification the piece, in a plastic tissue cassette, should be washed in running tap water for 3 hours to remove the acids. Prolonged washing results in swelling or expulsion of osteocytes and dislodgement of cells adjacent to the trabecular surface.
5. If the piece is to be sectioned immediately it can be held temporarily in water, otherwise it should be stored in 10% buffered formaldehyde or 70% alcohol. If the piece is to be paraffin embedded, store in 70% alcohol (either isopropyl or ethyl) until processing.
6. Pieces to be stored for greater than a month, and pieces that have been cut on the freezing microtome and are not needed immediately can be stored in 10% buffered formaldehyde.
7. Pieces stored in formaldehyde should be rinsed in tap water before using.

USE OF THE FREEZING MICROTOME

The freezing microtome used in this study was an Americal Optical (AO) 860 sliding microtome. Several proprietary manuals give details on the maintenance and use of this instrument (Richards, 1959; 1971; Anon, n.d.); and these should be consulted to get a general orientation of the instrument.

For use with tissue that is frozen, rather than parafin embedded, the instrument can be fitted with a variety of cooling units; some function by releasing CO<sub>2</sub> under pressure at the stage and near the knife. Others cool the stage directly with a connection to a condenser. The former method enables better control of the temperature, but the latter is less expensive and was available for the present work.

## PROCEDURE:

1. Place several drops of embedding medium for frozen sectioning (page 59) on the freezing stage and place the decalcified piece in this fluid, with the surface to be cut uppermost. Turn on the freezing unit, and hold the piece down against the stage to get a good seal while it is freezing (this usually takes about half a minute).
2. The tissue is frozen when a fine coat of frost appears on its surface and it turns a lighter color.
3. The upper surface of the tissue should be warmed prior to cutting; this reduces the friction on the blade, making it easier to cut. With the index finger or thumb rub the surface until it feels clammy to the touch. (Note: Thawing the tissue like this causes it to expand and often results in sections being thicker than indicated on the microtome setting.)

4. Two or three 30  $\mu\text{m}$  sections can be cut without rewarming the tissue. If thicker sections are needed the tissue should be warmed between each cut. We were able to cut, with good success, sections from 10 to 90  $\mu\text{m}$  thick, and 30  $\mu\text{m}$  gave the best results.
5. If properly done, the section should be intact, but end up as a crumpled mass or tight roll on the bevel of the knife. Use a finger tip carefully in a wiping motion, down the bevel of the knife from back to cutting edge, to transfer the section from the knife to a container of tepid tap water. The section will spread out or unroll as it warms to room temperature. A camel hair brush can be used to facilitate the spreading.
6. Select the best sections and transfer them one at a time to a tissue cassette. This is done by opening the cassette and floating the section into it using a camel hair brush. A small label can also be inserted if desired. The sections are stored in the cassettes in water until staining. We prefer metal tissue cassettes (page 59) at this stage because they sink and take up less volume, making it easier to process sections through staining; however plastic cassettes can be used. Metal cassettes should not be used in acid solutions.
7. The tissue cassette is used to carry the section through the entire staining procedure. For staining it is possible to put two or three sections in each cassette.

PARAFFIN PROCESSING

Sectioning tissues embedded in paraffin is conventional in most histologic labs. There are numerous good text books on the subject (e.g. Culling, 1974; Drury and Wallington, 1973; Sheehan and Hrapchak, 1980). Below is a brief outline including only the major points.

NOTE: where ever "alcohol" is indicated either isopropyl  $[(\text{CH}_3)_2\text{CHOH}]$  or ethyl  $[\text{CH}_3\text{CH}_2\text{OH}]$  can be used. The latter, abrevied "EtOH", is of better quality but more expensive. The use of EtOH is most critical in stains.

## SOLUTIONS:

- |  |          |   |
|--|----------|---|
| A. <u>100% "Absolute" Alcohol</u>                          |          |   |
| 100% alcohol   |          | * |
| B. <u>95% Alcohol</u>                                      |          |   |
| 100% alcohol   | 950.0 ml |   |
| Distilled Water  | 50.0 ml  |   |
| C. <u>80% Alcohol</u>                                      |          |   |
| 100% alcohol   | 800.0 ml |   |
| Distilled Water  | 200.0 ml |   |
| D. <u>Xylene</u> ( $\text{C}_6\text{H}_4(\text{CH}_3)_2$ ) |          | * |
| E. <u>Parafin wax</u> (see page 59)                        |          | * |

\* Quantities of these solutions depend on what quantities of material are being processed and whether the processing is done by hand or automatically.

## PROCEDURE:

1. Store decalcified pieces in 70% alcohol until processing.
2. Process as follows (dehydrate in alcohol [a-d], clear [e] and infiltrate [f]):

the tissue from excessive changes in surface tension which would destroy or detach cellular structures, but in the end allows water soluble stains to penetrate the cells

- a. Xylene: 2 changes, 5 minutes each.
- b. 100% alcohol: 2 changes, 3 minutes each.
- c. 95% alcohol: 2 changes, 2 minutes each.
- d. 80% alcohol: 1 change for 2 minutes.
- e. Into running tap water.

7. Stain:

STAINING TECHNIQUE

There are a multitude of stains used in histological work. Compiled with tremendous variation of active dyes, mordants, and other compounds to which tissues can be exposed is a seemingly endless variety of procedural and fine differences involving solution strengths and times; also small modifications and substitutions to recipes are often made, but unexplained or described, by individual laboratories and histologists. Hence, simply naming a conventional stain and assuming that it is used in a standard recipe and procedure, is inadequate for a full comprehension of the technique used. To reduce this problem and to make available a complete compendium of techniques, we list here relevant staining procedures.

To avoid redundancy, procedures or solutions that are commonly repeated with different stains are listed in detail once in this introductory section and then referred to in any of the following sections where appropriate.

Because we find frozen sections to be acceptable and more quickly, easily, and cheaply produced, the following discussions detail the steps necessary for treating frozen sections. The additional steps needed when staining sections embedded in paraffin are included in order of occurrence within parentheses and signalled with "P.", e.g., "P. (Deparaffinize and hydrate)".

### HEMATOXYLIN STAINS

A dye derived from the heartwood of the Neotropical hardwood tree Hematoxylon campechianum was one of the first stains used in histology (Humason, 1962:105). This is called "hematoxylin" because it is blood-colored and acidic. Not surprisingly, there are many variations in techniques employing this stain: we report here 3 common preparations: Ehrlich's, Harris', and Mayer's; Ehrlich's was preferable. Because precipitate particles commonly occur in dye solutions, all stains, especially hematoxylin, should be filtered immediately before being used.

Hematoxylin is categorized as either "progressive" or "regressive" stains: Progressive stains automatically increase in depth of color to a point where they can be used without further manipulations of stain. Regressive stains are used by over-staining the tissue, and then stain is removed by a destaining procedure.

### - BLUING SOLUTIONS

Once tissue has been stained with the basophilic hematoxylin, the color can be intensified, or blued. One of three solutions is commonly used: ammonia (vapors or solution), lithium carbonate, and running tap water. The first requires only a few quick dips in the solution, the second requires approximately 10 to 15 seconds of exposure, and the last needs a long exposure and should be adjusted to pH to prevent fading of the stain after years; it is not only slow but mild in effect. We used lithium carbonate to blue. Ammonia, while it is effective at enhancing the stain, is harsh and often removes both paraffin and frozen sections from the slides; it is also inconvenient to use because of the vapors.

## LITHIUM CARBONATE BLUING SOLUTION

## SOLUTIONS:

A. Saturated Lithium Carbonate Bluing Solution:

Lithium carbonate powder ( $\text{Li}_2\text{CO}_3$ )	> 1.3 g
Tap water	100.0 ml.

-COUNTERSTAINS

Back stain or counterstain can be used with all of the hematoxylin to contrast with the major effects of the hematoxylin. Eosin has been used for centuries as a counterstain; and there are many variants in its preparation and use. We will discuss only eosin-phloxine; other counterstain are described in histological texts (eg. Culling, 1974; Drury and Wallington, 1973; Humason, 1962; Luna, 1980; Sheehan and Hrapchak, 1980).

## EOSIN-PHLOXINE SOLUTION

## SOLUTIONS:

A. 1% Stock Alcoholic Eosin:

Eosin Y (water soluble)	1.0 g
Distilled water	20.0 ml
EtOH 95%	80.0 ml

Dissolve eosin in water and add alcohol. Keep tightly stoppered.

B. Stock Phloxine:

Phloxine B	1.0 g
Distilled water	100.0 ml

C. Working Eosin-Phloxine Solution:

Stock Alcoholic Eosin (A)	100.0 ml
Stock Phloxine (B)	10.0 ml
EtOH 95%	780.0 ml
Glacial acetic acid ( $\text{CH}_3\text{COOH}$ )	4.0 ml

Although the eosin-phloxine counter-stain was found to enhance the appearance of sections, it made no difference to the visibility of "lines of arrest" or "reversal lines" studied in age-determination work. Hence, the use of this counter-stain was not critical. If the eosin-phloxine is to be used, it is best to dehydrate the section in 80% alcohol beforehand. This dehydration step is unnecessary if this counter-stain is not used.

## EHRlich'S HEMATOXYLIN

## SOLUTIONS:

A. Ehrlich's Hematoxylin Solution:

Hematoxylin crystals	4.0 g
EtOH 95%	200.0 ml
Aluminum potassium sulfate ( $\text{AlK}(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$ )	6.0 g
Distilled water	200.0 ml
Glycerin ( $\text{C}_3\text{H}_8\text{O}_3$ )	200.0 ml
Glacial acetic acid ( $\text{CH}_3\text{COOH}$ )	20.0 ml
Sodium iodate ( $\text{NaIO}_3$ )	0.3 g

Dissolve the hematoxylin in the alcohol, then add the remaining ingredients. With the addition of sodium iodate the stain may be used immediately (Drury and Wallington, 1973). It is also recommended that this stain be aged, while exposed to sunlight, for three months, but this does not always work; sodium iodate is not necessary if natural aging is allowed. Sodium citrate ( $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ ) can be substituted for sodium iodate.

B. Lithium Carbonate Bluing Solution (see page 31)C. Eosin-Phloxine Working Solution (see page 31)

STAINING PROCEDURE: (Total time > 16 minutes).

## P. (Deparaffinize and hydrate to tap water).

1. Wash in running tap water for 1 min.
2. Stain in Ehrlich's hematoxylin solution (A); staining time will vary inversely with the thickness of the section e.g. 6  $\mu\text{m}$  at 10 min. and 60  $\mu\text{m}$  at 4 min.
3. Wash in running tap water for 2 min.
4. Blue in lithium carbonate (B) for about 10 seconds (3-4 dips).

## EHRlich'S HEMATOXYLIN (cont.)

5. Wash in running tap water for 2 min.

Optional steps 6 and 7-

6. Dehydrate in 80% alcohol for 2 min; and

7. Counter-stain in eosin-phloxine solution (C) for 1.5 min.

P. (Dehydrate and clear, see page 55).

8. Mount (see page 55).

## RESULTS:

(From Drury and Wallington, 1973, page 127)

Stains mucopolysaccharide substances such as cartilage and the cement lines of bones dark blue. Areas of calcification will also be stained intensely blue.

In all hematoxylin-eosin staining (for Harris', Mayer's and Ehrlich's):

Nuclei - blue to blue black.

Cartilage - pink or light blue to dark blue depending on type and the stain used, being darkest with Ehrlich's.

Cement lines of bones - blue with Ehrlich's.

Calcium and calcified bones - purplish blue.

Muscle fibers, thick elastic fibers, decalcified bone matrix - deep pink.

Collagen and osteoid tissue - light pink.

## REFERENCE:

Luna, 1980: 35.

## HARRIS' HEMATOXYLIN

## SOLUTIONS:

A. Harris's Hematoxylin Solution:

Hematoxylin crystals	5.0 g.
EtOH 100%	50.0 ml.
Aluminum potassium sulfate ( $\text{AlK}(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$ )	100.0 g.
Distilled water	1000.0 ml.
Mercuric oxide (red) ( $\text{HgO}$ )	2.5 g.

Dissolve the hematoxylin in the alcohol. Dissolve the aluminum potassium sulfate in the water by heating. Remove from heat and mix the two solutions. Bring to a boil as rapidly as possible (but limit the heating to less than 1 minute and stir often). Remove from heat and slowly add the mercuric oxide. Reheat to a simmer until the solution becomes dark purple, remove from heat immediately and plunge the vessel into cold water until cool. The stain is ready to use when cool. Filter before use. The occurrence of a metallic sheen on the surface indicates that the stain is good.

B. Acid-Alcohol Solution:

EtOH 70%	1000.0 ml.
Hydrochloric Acid ( $\text{HCl}$ )(Concentrated, 37%)	10.0 ml.

C. Lithium Carbonate Bluing solution (see page 31)D. Eosin-Phloxine Working Solution (see page 31)

STAINING PROCEDURE: (Total time > 21 minutes)

P. (Deparaffinize and hydrate to tap water).

1. Wash in running tap water for 2 min.

2. Stain in Harris' hematoxylin (A): staining time will vary

## HARRIS' HEMATOXYLIN (cont.)

inversely with the thickness of the section, eg. 6 um at 12 min., 30 um at 10 min. and 60 um at 4 min.

3. Wash in running tap water for 2 min.
4. Differentiate (destain) in acid-alcohol solution (B), 3-4 dips. Differentiation can be checked under a microscope, and continued if necessary.
5. Wash 3 min. in water.
6. Blue in lithium carbonate (C) for about 10 seconds (3 - 4 dips).
7. Wash in running tap water for 3 min.

Optional steps 8 and 9.

8. Dehydrate in 80% alcohol for 2 min.; and
9. Counter-stain in eosin-phloxine solution (D) for 1.5 min.
- P. (Dehydrate and clear, see page 55).
10. Mount (see page 55).

## RESULTS:

See Ehrlich's hematoxylin.

## REFERENCE:

Luna, 1980:34, modified by Bonshock.

## MAYER'S HEMATOXYLIN

## SOLUTIONS:

A. Mayer's Hematoxylin Solution:

Hematoxylin crystals	1.0 g
Distilled water	1000.0 ml
Sodium iodate ( $\text{NaIO}_3$ )	0.2 g
Aluminum potassium sulfate ( $\text{AlK}(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$ )	50.0 g
Citric acid ( $\text{C}_6\text{H}_8\text{O}_7$ )	1.0 g
Chloral hydrate ( $\text{C}_2\text{H}_3\text{Cl}_2\text{O}_2$ )	50.0 g

Dissolve the aluminum potassium sulfate in water, without heat; add hematoxylin crystals. Add sodium iodate, chloral hydrate, and the citric acid, shake until all components are completely dissolved. The stain keeps well for months.

B. Lithium Carbonate Bluing Solution (see page 31)C. Eosin-Phloxine Working Solution (see page 31)

## STAINING PROCEDURE: (Total time &gt; 21 minutes)

## P. (Deparaffinize and hydrate to tap water).

1. Wash in running tap water for 1 min.
2. Stain in Mayer's hematoxylin solution (A) for 15 min.
3. Rinse off excess stain in tap water.
4. Blue in lithium carbonate solution (B) for about 10 seconds (3-4 dips).
5. Wash in running tap water for 5 min.

## Optional steps 6 and 7.

6. Dehydrate in 80% alcohol for 2 min.; and
  7. Counter-stain in eosin-phloxine (C) for 1.5 min.
- P. (Dehydrate and clear, see page 55).
8. Mount (see page 55).

MAYER'S HEMATOXYLIN (cont.)

RESULTS:

See Ehrlich's hematoxylin

REFERENCE:

Luna , 1980:33.

### SPECIAL STAINS

In addition to the conventional hematoxylin stains, there is a multitude of special stains, which have been developed to emphasize certain features. Alizarin Red S stains for calcium; the intensity of the stain is an indicator of calcium density, which might change in relation to lines of arrest. Alcian Blue stains for mucosubstrates, which are thought to be concentrated at lines of arrest. Aldehyde fusion stains for sulfonated acid mucopolysaccharydes, also thought to be concentrated at lines of arrest. Gordon and Sweet's stain was developed to emphasize cement lines. Manuel's method was developed especially to demonstrate reticular structures. Masson's Trichrome produces three colors, of which blue is specific to collagen fibers, which may show large changes in density near lines of arrest.

In general, these stains involve considerably more time, labor, solutions, and expense than do the standard hematoxylin stains. Curiously, in addition to these drawbacks we found results from the special stains to be less useful than those from the more simple hematoxylin stains. However, they are included here for the sake of completeness, and in the hope that someone in the future may be able to get better results from them.

The procedures outlined here are meant for use with 6 um sections; staining of thicker sections may necessitate modifications in staining times.

## ALIZARIN RED S

## SOLUTIONS:

A. 0.1% Ammonium Hydroxide

Ammonium hydroxide (NH <sub>4</sub> OH) (28%)	0.1 ml
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Distilled water	100.0 ml
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B. 1% Alizarin Red S Solution

Alizarin Red S	1.0 g
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Distilled water	100.0 ml
-----------------	----------

0.1% ammonium hydroxide (A)	10.0 ml
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Stir the Alizarin into the distilled water so that only a few small grains of the dye remain undissolved. Adjust the pH of this solution to between 6.36 and 6.40 with the ammonium hydroxide. This solution is stable for one month.

## STAINING PROCEDURE: (Total time &gt; 11 minutes).

P. (Deparaffinize and hydrate to water).

1. Stain in 1% Alizarin Red S (B) for 10 minutes.

2. Rinse in distilled water.

P. (Dehydrate and clear, see page 55).

3. Mount (see page 55).

## RESULTS:

Calcium salts - stain intense reddish-orange.

## REFERENCE:

Dahl, L. K. 1952. J. Exp. Med. 95: 474-479, cited in Luna, 1980: 175, 176.

## MASSON'S TRICHROME METHOD

## SOLUTIONS:

A. Bouin's Solution

Picric acid ( $C_6H_2(NO_2)_3OH$ )	1.22 g
Distilled water	100.0 ml
Formaldehyde (HCHO) (Commercial stock 37-40%)	33.3 ml
Glacial acetic acid ( $CH_3COOH$ )	6.7 ml

B. Weigert's Iron Hematoxylin: Solution I

Hematoxylin crystals	1.0 g
EtOH 95%	100.0 ml

C. 29% Aqueous Ferric Chloride

Ferric Chloride ( $FeCl_3 \cdot 6 H_2O$ )	1.5 g
Distilled water	5.0 ml

D. Weigert's Iron Hematoxylin: Solution II

29% Aqueous ferric chloride (C)	4.0 ml
Distilled water	95.0 ml
Hydrochloric acid (HCl) concentrated	1.0 ml

E. Weigert's Iron Hematoxylin: Working Solution

(equal parts of Solution I (B) and Solution II (D)).

F. 1% Aqueous Biebrich Scarlet

Biebrich scarlet	1.0 g
Distilled water	100.0 ml

G. 1% Aqueous Acid Fuchsin

Acid fuchsin	1.0 g
Distilled water	100.0 ml

H. Biebrich Scarlet-Acid Fuchsin Solution

1% Aqueous Biebrich scarlet (F)	90.0 ml
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## MASSON'S TRICHOME METHOD (cont.)

1% Aqueous acid fuchsin (G)	10.0 ml
Glacial acetic acid (CH <sub>3</sub> COOH)	1.0 ml
<u>I. Phosphomolybdic-Phosphotungstic Acid Solution</u>	
Phosphomolybdic acid (P <sub>2</sub> O <sub>5</sub> .24 MoO <sub>3</sub> .x H <sub>2</sub> O)	5.0 g
Phosphotungstic acid (P <sub>2</sub> O <sub>5</sub> .24 WO <sub>3</sub> .x H <sub>2</sub> O)	5.0 g
Distilled water	200.0 ml
<u>J. Aniline Blue Solution</u>	
Aniline blue	2.5 g
Glacial acetic acid (CH <sub>3</sub> COOH)	2.0 ml
Distilled water	100.0 ml
This solution has a tendency to become weak unexpectedly, and a fresh stock may be needed.	
<u>K. 1% Glacial Acetic Acid Solution</u>	
Glacial acetic acid (CH <sub>3</sub> COOH)	1.0 ml
Distilled water	100.0 ml

## STAINING PROCEDURE: (Total time &gt; 48 minutes)

- P. (Deparaffinize and hydrate to distilled water).
1. Mordant in Bouin's (A) for 1 hour at 56° C or overnight at room temperature, approx 20°C if specimen is fixed in formaldehyde.
  2. Wash in running water.
  3. Rinse in distilled water.
  4. Stain in Weigert's iron hematoxylin solution (E) for 10 minutes.
  5. Wash in running water for 10 minutes.
  6. Rinse in distilled water.
  7. Stain in Biebrich scarlet-acid fuchsin solution (H) for 2 minutes (save solution).

## MASSON'S TRICHROME METHOD (cont.)

8. Rinse in distilled water.
9. Differentiate (destain) collagen in Phosphomolybdic-phosphotungstic acid solution (I) for 15 minutes (discard solution).
10. Stain in anilin blue solution (J) for 5 minutes (save solution).
11. Rinse in distilled water.
12. 1% acetic acid solution (K) for 3 to 5 minutes (discard solution).
- P. (Dehydrate and clear, see page 55).
13. Mount (see page 55).

## RESULTS:

Nuclei	- black
Cytoplasm, keratin, muscle fibers and intercellular fibers	- red
Collagen	- blue

## NOTES TO VARIOUS PROCEDURAL STEPS:

1. Formaldehyde can be used in place of Bouin's.
2. Wash at step 2 needs to last until yellow color disappears.
4. Stains nuclei black.
7. Stains cytoplasm and collagen red.
9. Destain collagen; check to be sure that it is colorless before going to next step.
10. Check after finishing to be sure fine collagen fibers are stained, if not repeat with fresh anilin blue solution.

## REFERENCE:

Masson, P.J. 1929. J. Techn. Methods 12: 75-90, AFIP modification;  
Luna, 1980: 94-95.

NOTE: Some histologists prefers Gomori trichrome, which is more rapid, but less controlable.

## MANUEL'S METHOD FOR RETICULUM

## SOLUTIONS:

A. 1% Uranium Nitrate Solution

Uranium nitrate ( $\text{UO}_2(\text{NO}_3)_2 \cdot 6 \text{H}_2\text{O}$ )	1.0 g
Distilled water	100.0 ml

B. Ammoniacal Silver Solution

Distilled water	1000.0 ml
Silver nitrate ( $\text{AgNO}_3$ )	100.0 g
Ammonium hydroxide ( $\text{NH}_4\text{OH}$ ) 58 % (28% ammonia gas by assay)	60.0 ml

In 1000 ml of distilled water dissolve 100 gm of silver nitrate. Pour off 70 ml and save in a separate container. To the 930 ml of 10% silver nitrate add 60 ml of 58% ammonium hydroxide, shaking the flask vigorously (solution should become dark then clear; if not, continue adding ammonium hydroxide drop by drop until it does clear). Of the 70 ml of 10 % silver nitrate saved, add 50 ml slowly to the ammonium hydroxide silver nitrate mixture. The solution should become slightly cloudy, if it does not, add part or all of the remaining 20 ml of the silver nitrate. Let the solution stand overnight and filter before using. Store in the refrigerator and use as needed.

C. 1% Formaldehyde solution

Formaldehyde ( $\text{HCHO}$ ) (commercial stock, 37-40%)	1.0 ml
Distilled water	100.0 ml

D. 1% Gold Chloride Solution

Gold chloride ( $\text{AuCl}_3 \cdot \text{HCl} \cdot 4 \text{H}_2\text{O}$ )	1.0 g
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## MANUEL'S METHOD FOR RETICULUM (cont.)

	Distilled water	100.0 ml
E.	<u>5% Sodium thiosulfate (Hypo) Solution</u>	
	Sodium thiosulfate ( $\text{Na}_2\text{S}_2\text{O}_3$ )	5.0 g
	Distilled water	100.0 ml
F.	<u>5% Aluminum sulfate solution</u>	
	Aluminum sulfate ( $\text{Al}_2(\text{SO}_4)_3 \cdot 18 \text{H}_2\text{O}$ )	5.0 g
	Distilled water	100.0 ml
G.	<u>Nuclear Fast Red (Kernechtrot) Solution</u>	
	Nuclear fast red 5%	0.1 g
	Aluminum sulfate solution (F)	100.0 ml
	Thymol ( $\text{C}_{10}\text{H}_{14}\text{O}$ )	1 grain

Dissolve 0.1 gm nuclear fast red in 100 ml of 5% solution of aluminum sulfate (F) with the aid of heat. Cool, filter, and add one grain of thymol as a preservative.

## STAINING PROCEDURE: (Total Time &gt; 15 min.)

- P. Deparaffinize and hydrate to distilled water.
1. Sensitize in uranium nitrate solution (A) for 2 minutes.
  2. Dip quickly in running tap water.
  3. Impregnate in ammoniacal silver solution (B) for 3 minutes.
  4. Wash in running water, two or three quick dips (until no more white precipitate appears in the water).
  5. Develop in formaldehyde solution (C) for 1 minute.  
Repeat steps 3-5 once before proceeding to step 6.
  6. Wash in running tap water.
  7. Tone in gold chloride solution (D) for 1 minute.
  8. Wash in running tap water.

MANUEL'S METHOD FOR RETICULUM (cont.)

9. Reduce in hypo (E) for 1 minute.
10. Wash in running water.
11. Counterstain with Kernechtrot solution (G) for 5 minutes.
12. Rinse in distilled water for three changes.
- P. (Dehydrate and clear, see page 55).
13. Mount (see page 55).

NOTES TO VARIOUS PROCEDURAL STEPS:

4. If sections fail to get sufficiently dark in the reducer (step 5), decrease the time in wash (step 4). If an excessive precipitate forms in the reducer (step 5) increase time in the wash (step 4).
4. Reticulum turns black and other structures, pink to grey pink.

RESULTS:

Reticulum                    - black  
Nuclei and background - red

REFERENCE:

Stain developed by Mr. Benedicto Manuel, Jr. AFIP, see Luna, 1980:  
89-90.

## GORDON AND SWEET'S METHOD FOR CEMENT LINES

## SOLUTIONS:

A. 0.5% Potassium Permanganate Solution:

Potassium permanganate ( $\text{KMnO}_4$ )	0.5 g
Distilled water	100.0 ml

B. 3% Sulfuric Acid

Sulfuric acid ( $\text{H}_2\text{SO}_4$ )	3.0 ml
Distilled water	100.0 ml

C. Acidified Permanganate Solution:

0.5% potassium permanganate (A)	48.0 ml
3.0% sulfuric acid (B)	2.0 ml

(This solution is stable for 48 hours)

D. 1% Oxalic Acid Solution

Oxalic acid ( $\text{H}_2\text{C}_2\text{O}_4 \cdot 2 \text{H}_2\text{O}$ )	1.0 g
Distilled water	100.0 ml

E. 2.5% Ferric Ammonium Sulfate

Ferric ammonium sulfate ( $\text{Fe}(\text{NH}_4)(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$ )	2.5 g
Distilled water	100.0 ml

F. 10% Silver Nitrate Solution

Silver nitrate ( $\text{AgNO}_3$ )	5.0 g
Distilled water	100.0 ml

G. 8% Sodium Hydroxide

Sodium hydroxide ( $\text{NaOH}$ )	8.0 g
Distilled water	100.0 ml

H. Diamine Silver Hydroxide Solution

10% silver nitrate solution (F)	10.0 ml
8% sodium hydroxide (G)	10.0 ml

## GORDON AND SWEET'S METHOD FOR CEMENT LINES (cont.)

Ammonium hydroxide (NH <sub>4</sub> OH) 28%	10.0 ml
Distilled water	80.0 ml

To 10 ml of 10% silver nitrate solution, add 28% ammonium hydroxide drop by drop. A precipitate will form, but continue to add ammonium hydroxide, shaking vigorously until the precipitate disappears. Then add 10 ml of 8% sodium hydroxide. Again add ammonium hydroxide and dissolve the precipitate in the above manner. Add distilled water to total 100 ml of solution. Filter and store immediately in a brown bottle. The solution remains stable for months when refrigerated.

I. 10% Formaldehyde Solution

Formaldehyde (HCHO)(commercial grade, 37-40%)	100.0 ml
Distilled water	900.0 ml

J. 1% Gold Chloride Solution

Gold chloride (15 grains) (Au Cl <sub>3</sub> .HCl.4 H <sub>2</sub> O)	1.0 g
Distilled water	100.0 ml

Break glass vial and empty contents (15 grains) into graduate cylinder with 100 ml of distilled water.

K. 0.2% Gold Chloride Solution

1% Gold chloride solution (J)	10.0 ml
Distilled water	40.0 ml

(Solution is very stable and may be used repeatedly; keep refrigerated.)

L. 5% Sodium Thiosulfate Solution

Sodium thiosulfate (Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> )	5.0 g
Distilled water	100.0 ml

M. Mayer's hematoxylin solution (see page 37 ).

## GORDON AND SWEET'S METHOD FOR CEMENT LINES (cont.)

## STAINING PROCEDURE: (Total time &gt; 45 min.)

- P. (Deparaffinize and hydrate to distilled water).
1. Oxidize in acidified permanganate solution (C) for 1 minute.
  2. Wash in distilled water for 2 minutes.
  3. Bleach in 1% oxalic acid (D) for 1 minute.
  4. Wash in tap water for 2 minutes.
  5. Wash in 2 changes of distilled water, 1 minute each.
  6. Mordant in 2.5% ferric ammonium sulfate (E) for 1 minute.
  7. Wash in tap water for 2 minutes, followed with 2 changes of distilled water for 30 seconds each.
  8. Permeate with the diamine silver solution (H) for 1 minute.
  9. Rinse in distilled water for 20 seconds.
  10. Reduce in 10% formalin (I) for 3 minutes.
  11. Wash in tap water for 3 minutes.
  12. Tone in 0.2% gold chloride (K) for 10 minutes.
  13. Wash in tap water.
  14. Wash for 5 minutes in 5% sodium thiosulfate (L).
  15. Wash in tap water for 3 minutes.
  16. Stain in Mayer's hematoxylin (M) for 1 minute.
  17. Wash in tap water and then rinse in distilled water.
- P. (Dehydrate and clear, see page 55).
18. Mount (see page 55).

## RESULTS:

Bone-bluish gray with well-defined unstained cement lines.

Reticulum fibers - black

GORDON AND SWEET'S METHOD FOR CEMENT LINES (cont.)

REFERENCE:

Dury and Wallington, 1973: 170-171 and Sheehan and Hrapchak, 1980:  
104.

NOTE: Originally designed for undecalcified, plastic embedded sections.

## ALDEHYDE FUCHSIN

## SOLUTIONS:

A. Aldehyde Fuchsin Solution:

Basic Fuchsin	1.0 g
EtOH 80%	200.0 ml
Normal Hydrochloric acid (HCl)	30.0 ml
Paraldehyde (CH <sub>3</sub> CHO) <sub>3</sub>	3.0 ml

Disolve the basic Fuchsin in 200 ml of 80% EtOH. Add 30 ml of HCl followed by 3 ml of paraldehyde. Store this solution in a dark, tightly stoppered bottle, and put it in a dark place overnight, or for at least four hours. When the solution assumes a deep violet or purplish violet color, it is ready for use. Store the stain in a refrigerator; it is good for 4 or 5 months.

B. Saturated Aqueous Picric Acid:

Picric acid (C <sub>6</sub> H <sub>2</sub> (NO <sub>2</sub> ) <sub>3</sub> OH)	1.22 g
Distilled water	100.0 ml

C. Van Gieson Solution:

Acid Fuchsin, 1% aqueous	2.5 ml
Saturated aqueous picric acid	97.5 ml

STAINING PROCEDURE: (Total time > 12 hours).

- P. (Deparaffinize and hydrate to tap water)
1. Wash in running tap water for 2 minutes.
  2. Stain for several hours or overnight in the aldehyde fuchsin solution (A).
  3. Rinse in 3 or 4 changes of 95% EtOH until the sections are clear and the color ceases to run.

## ALDEHYDE FUCHSIN (cont.)

4. Rinse in tap water
5. Stain for 10 seconds up to 2 minutes in Van Gieson Solution (C).  
P. (Dehydrate and clear, see page 55).
6. Mount (see page 55).

## RESULTS:

Chondroitin sulfuric acid of cartilage deeply stained; that present in heart valves, umbilical cord, tendon and skin less deeply stained. Sharp delineation of elastic tissue, sulfonated mucopolysaccharides of blood vessels. Granules of mast cells Beta in pancreatic islets and adenochoyphysis.

## REFERENCE:

S. K. Abul Haj, AFIP handout; modified from Luna 1980: 166.

## ALCIAN BLUE METHOD FOR MUCOSUBSTANCES pH 2.5

## SOLUTIONS:

A. 3% Acetic Acid Solution

Glacial acetic acid ( $\text{CH}_3\text{COOH}$ )	3.0 ml
Distilled water	97.0 ml

B. 1% Alcian Blue Solution

Alcian Blue, 8 GX	1.0 g
3% Acetic acid (A)	100.0 ml
Thymol ( $\text{C}_{10}\text{H}_{14}\text{O}$ )	1 grain

Adjust pH to 2.5, filter and add one grain of thymol.

C. Nuclear Fast Red (Kernechtrot) Solution

Aluminium sulfate ( $\text{Al}_2(\text{SO}_4)_3 \cdot 18 \text{H}_2\text{O}$ )	5.0 g
Distilled water	100.0 ml
Nuclear fast red	0.1 g
Thymol ( $\text{C}_{10}\text{H}_{14}\text{O}$ )	1 grain

Dissolve the aluminium sulfate in water; with the aid of heat dissolve the nuclear fast red in 5% solution of aluminium sulfate. Cool, filter, and add one grain of thymol as a preservative.

## STAINING PROCEDURE: (Total time &gt; 50 minutes).

- P. (Deparaffinize and hydrate to distilled water).
1. Mordant in acetic acid solution (A) for 3 minutes.
  2. Stain in Alcian blue solution (B) for 30 minutes.
  3. Wash in running water for 10 minutes.
  4. Rinse in distilled water.
  5. Counterstain in Kernechtrot solution (C) for 5 minutes.
  6. Wash in running water for 1 minute.

## ALCIAN BLUE METHOD FOR MUCOSUBSTANCES pH 2.5 (cont.)

P. (Dehydrate and clear, see page 55).

7. Mount (see page 55).

## RESULTS:

Weakly acidic sulfated mucosubstances, hyaluronia acid and sialomucins-stains dark blue. Nuclei stain weakly in formalin-fixed tissue if Kernechtrot is not used as counterstain.

## REFERENCE:

Lev, R. and S. S. Spicer. 1964. J. Histicem. Cytochem. 12:309, cited in Luna 1980: 163-164.

MOUNTING (or COVER SLIPPING)

Once a section has been prepared for examination it is best preserved for future work by covering it, and to hold down the cover slip a mounting medium is used. Normally this is a resinuous compound, and these materials are not water soluble but soluble in xylene or toluene. Sections must be dehydrated before mounting in resins, but dehydration results in the distortion and shrinkage of sections, especially when they are thicker than the normal 6 um used in histology. The less distortion a section experiences the more meaningful are the measurements taken from it in interpreting rates of growth, rates of remodeling, and relative amounts of lamellar bone available with readable growth layers. Off course, paraffin embedded sections have already been subjected to shrinkage and distortion (commonly as much as 10 to 15% shrinkage occurs), so there is no <sup>ad</sup>isvantage with mounting in resin.

Mounting in glycerine ( $C_3H_8O_3$ ), a water miscible compound, produces very little distortion, and although the mounting process is more involved we prefer this because it gives less distorted sections with more usable information. We prefer this with thicker than normal (>6 um) frozen (undehydrated) sections. However, glycerin is subject to dehydration and results in fading of the stain.

## RESIN MOUNTING

## 1. Dehydrate and Clear

Once sections have been stained they are ready to be mounted. Most mounting media are soluble in xylene, but not water, so the sections must be dehydrated.

MOUNTING (or COVER SLIPPING) (cont.)

- a. 80% alcohol: 1 change for 1 minute.
- b. 95% alcohol: 2 changes, 2 minutes each.
- c. 100% alcohol: 2 changes, 2 minutes each
- d. Xylene: 2 changes, 2 or 3 minutes each.

Agitating the slides while in these solutions gives better differentiation, or contrast, in the stains.

2. Wipe away any surplus xylene on the slide with a clean cloth.
3. Without allowing the section to dry, place several drops of mounting medium on top of it.
4. Place a cover slip over the section, touching down one edge first. Push slightly on the top of the cover slip, toward one side, to force out air bubbles.
5. Surplus mounting medium can be removed by quickly immersing the slide into xylene and wiping off with a clean tissue or cloth.
6. Allow the mounting medium to dry while the slide lays flat for several hours.
7. Label the slide.

## GLYCERIN MOUNTING

1. Sections placed directly into glycerin ( $C_3H_8O_3$ ) from water will warp making it difficult to get them to lay flat on the slide; passing the section through a series of increasingly concentrated glycerine/water baths prevents this.
2. Set up two baths with increasing concentrations of glycerin in water, the first with a 1:1 solution; the second, 3:1. Stir the solutions periodically to prevent the glycerin from separating out from the water. Sections can be perfused with glycerine while still in the metal tissue cassettes.
3. Transfer the cassette from the last procedural step (eg. water wash bath) in the stain series to the 1:1 glycerine bath and from there to the 3:1 glycerine bath, soaking in each bath for 2 to 3 minutes.
4. With a camel hair brush place 2-3 drops of glycerine on a glass slide. Paint an area larger than the section to be mounted.
5. Remove the section from the cassette and using the brush float the section into the glycerine painted section of the slide. Remove the slide from the bath.
6. Place one to two drops of glycerine on top of the section. Coverslip the slide. Press down with slight pressure to remove air bubbles and excess water and glycerine.
7. Clean up excess water and glycerin with a piece of filter paper.
8. Seal the edges of the coverslip with clear fingernail polish. Should the section show signs of drying out, the fingernail polish can be removed with acetone ( $CH_3CO.CH_3$ ) and additional glycerine can be added.
9. Inspect the slide after 24 hours to ensure that all the edges are

properly sealed. The edges of the cover can be sealed an additional time now.

10. Label slide.

PRODUCTS MENTIONED IN TEXTPlastic Tissue Cassette:

Tissue-Tek III

Lab-Tek Division

Miles Laboratories, Inc.

Naperville, Ill. 60540

Small = Uni-Cassette # 4170

Large = Mega-Cassette # 4173

Embedding Medium for Frozen Sectioning:

O.C.T., Compound # 4583

Division Miles Laboratories, Inc.

Elkhart, Indiana 46514

or

Krystalor Mounting Medium # 64969

Harleco

Gibbstown, N.J. 08027

Refrigerant:

Cryokwick (IEC Catalogue N° 3377)

Damon/IEC Division

300 Second Avenue

Needham Hts, Mass. 02194

or

Gebauer's Skin Refrigerant (6505-00-576-8915)

Gebauer Chemical Co.

Cleveland, Ohio 44104

Microtome Knife Sharpening:

C. L. Sturkey

Mechanician

P.O. Box # 182

Perkiomenville, Penna. 18074

Tel. (215) 754-7296

Metal Tissue Cassette:

Lancer Biopsy Holder # 8889-240001

Lancer, Division of Sherwood Medical

St. Louis, Mo. 63103

Paraffin Wax:

Paraplast Tissue embedding medium # 60001

Sherwood Medical Industries Inc.

1831 Olive St.

St. Louis, Mo. 63103

LIST OF CHEMICALS

Acetone	$\text{CH}_3\text{CO}\cdot\text{CH}_3$
Aluminium potassium sulfate	$\text{AlK}(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$
Aluminium sulfate	$\text{Al}_2(\text{SO}_4)_3 \cdot 18 \text{H}_2\text{O}$
Ammonium hydroxide	$\text{NH}_4\text{OH}$
Chloral hydrate	$\text{C}_2\text{H}_3\text{Cl}_2\text{O}_2$
Citric acid	$\text{C}_6\text{H}_8\text{O}_7$
Ethyl alcohol (EtOH)	$\text{CH}_3\text{CH}_2\text{OH}$
Ferric ammonium sulfate	$\text{Fe}(\text{NH}_4)(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$
Ferric chloride	$\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$
Formaldehyde	$\text{HCHO}$
Formic acid	$\text{HCOOH}$
Glacial acetic acid	$\text{CH}_3\text{COOH}$
Glycerin	$\text{C}_3\text{H}_8\text{O}_3$
Gold chloride	$\text{AuCl}_3 \cdot \text{HCl} \cdot 4 \text{H}_2\text{O}$
Hydrochloric acid	$\text{HCl}$
Isopropyl alcohol	$(\text{CH}_3)_2\text{CHOH}$
Litium carbonate	$\text{Li}_2\text{CO}_3$
Oxalic acid	$\text{H}_2\text{C}_2\text{O}_4 \cdot 2 \text{H}_2\text{O}$
Paraldehyde	$(\text{CH}_3\text{CHO})_3$
Phosphomolybdic acid	$\text{P}_2\text{O}_5 \cdot 24 \text{MoO}_3 \cdot x \text{H}_2\text{O}$
Phosphotungstic acid	$\text{P}_2\text{O}_5 \cdot 24 \text{WO}_3 \cdot x \text{H}_2\text{O}$
Picric acid	$\text{C}_6\text{H}_2(\text{NO}_2)_3\text{OH}$
Potassium permanganate	$\text{KMnO}_4$
Silver nitrate	$\text{AgNO}_3$

LIST OF CHEMICALS (cont.)

Sodium citrate	$\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2 \text{H}_2\text{O}$
Sodium hydroxide	$\text{NaOH}$
Sodium iodate	$\text{NaIO}_3$
Sodium thiosulfate	$\text{Na}_2\text{S}_2\text{O}_3$
Sulfuric acid	$\text{H}_2\text{SO}_4$
Thymol	$\text{C}_{10}\text{H}_{14}\text{O}$
Xylene	$\text{C}_6\text{H}_4(\text{CH}_3)_2$
Uranium nitrate	$\text{UO}_2(\text{NO}_3)_2 \cdot 6 \text{H}_2\text{O}$

## PRELIMINARY RESULTS

Bones of more than 80 individuals of five species were sectioned producing more than 600 slides. These are still being studied, but the following comments are appropriate with the preliminary results: We have insufficient information to comment on methods of preparation; hand cleaning has been used because it is safest. However, this technique is very time consuming, for it may take almost one hour to hand clean the major long bones of one animal. For fixation we use 5 - 10% buffered formaldehyde, simply because it is convention, and because it gives acceptable results. Salt is not a good preservative for bones, and even after washing, there may be uneven staining. Air dried, however, specimens may give perfectly good results, although fixing in formaldehyde before sectioning may enhance contrast of growth layers.

The best bones to section are the humerus and femur, and the best place to section is at the greatest constriction of the shaft, or slightly proximal to it. Radiographs help in locating the most concentration of compact bone, with the greatest number of growth layers.

We prefer cutting a series of wafers and pieces with the diamond blade saw. Pieces about 3 - 4 mm thick are decalcified in hydroformic/hydrochloric acid solution. Thin sections cut at 30 um on the freezing microtome, stained in Ehrlich's hematoxylin, and mounted in glycerin gave best results with our equipment. However, paraffin embedded sections cut at 6 um, stained in Harris' hematoxylin and mounted in resin also gave good results when processed at AFIP: superior equipment, materials and expertise made this possible.

Bone sections of Dermochelys are very difficult to interpret. Lepidochelys is also difficult because of a large relative amount of spongy bone. The other species, especially Eretmochelys, often present very readable growth layers.

Despite earlier pilot studies there is not a simplistic way to interpret growth layers in sea turtle bones. Several factors confound this:

- 1- Bone is resorbed from the inside out, so older layers are progressively destroyed in the normal life of the bone. Hence, older growth layers and lines of arrest are simply not present.
- 2- Lamellar accretion is not equal on all places of the circumference; some surfaces increase rapidly in radial dimensions while other surfaces evidently do not expand. This is shown by lines of arrest which fuse in certain places and fan out in others. Hence, the number and conspicuousness of growth layers is a function of which radius of a cross section is examined.
- 3- "Bundle" bone occurs in certain places, and the rapid radial growth that occurs there totally disrupts any evidence of growth bands.
- 4- Individual lines of arrest show irregular form. In some individuals the lines did not trace circles or even ellipses, but doubled back, making loops, or wandered irregularly.
- 5- The visibility of individual lines of arrest is inconsistent; a dark contrasting line may fade entirely from view as it is traced across a section.
- 6- Widths of individuals growth layers show no predictable relationship. For no apparent reason a layer will be many times more (or less) wide than a neighboring layer.

7- Known-age specimens of green turtle from Cayman Turtle farm often show more growth layers than their age in years, but less than their age in months.

The following 17 figures illustrate some of these difficulties. Preliminary interpretation could be offered for annual or mensual nature of growth layers and lines of arrest in long bones of sea turtles. However, at this point in the study, the results are equivocal, and it would be unwise to draw conclusions.

Further work is need to elucidate the characteristics of lines of arrest:

What elemental or molecular features characterize these indicators of growth?

How can lines of arrest and growth layers be made most conspicuous for counting?

And, most critically, are these indicators of periodic growth phenomena?

And do they correspondent to a fixed unit of time?

What is that unit?

The answers will come only after detailed study of the materials prepared and of older, known-age materials now available.

Figure 1

Femur of 14 month old captive-reared Chelonia mydas (LC 199) showing two concentric lines (1 and 3) with a partial line between (2)



Fig 1

Figure 2

Humerus of 14-month old captive reared Chelonia mydas (LC 199) showing one concentric line

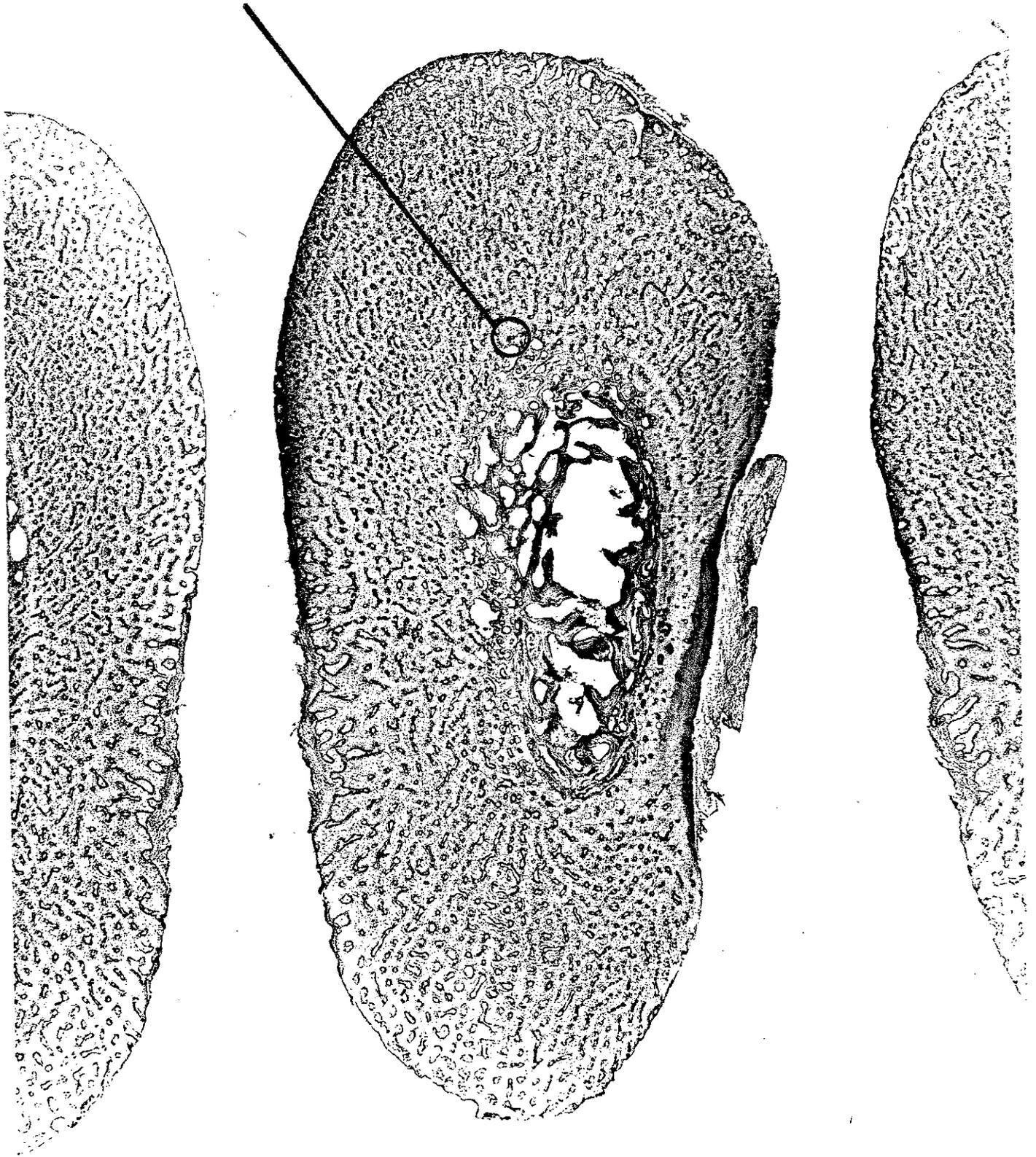


Fig 2

## Figure 3

Femur of 18-month old captive-reared Chelonia mydas (LC 200) showing three clear bands separated by two concentric lines (1 and 2), in an area of bundle bone line 2 becomes at least 3 clear lines.

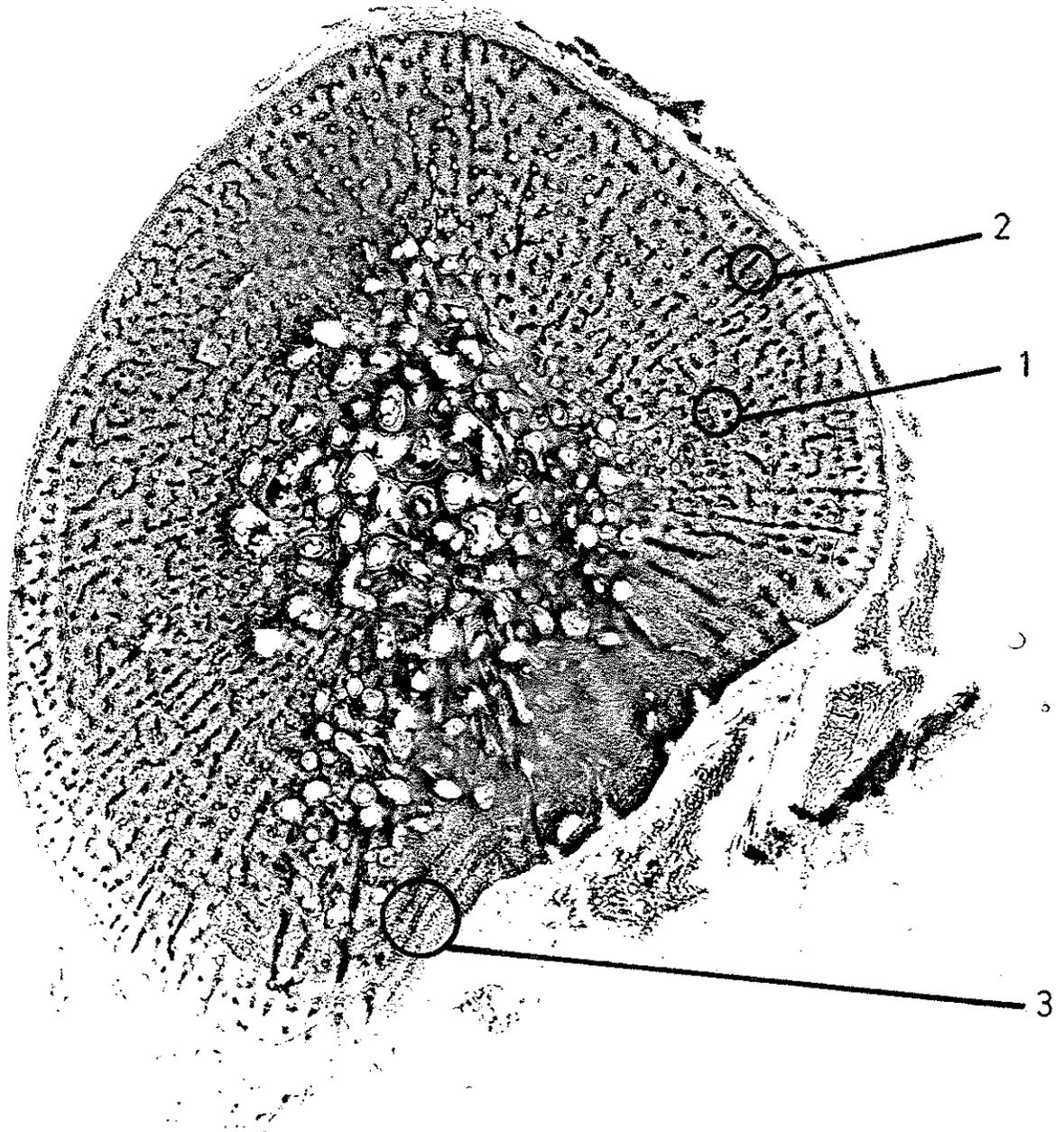


Fig 3

## Figure 4

Two sections of humerus of 18-month old captive-reared Chelonia mydas (LC 200) showing a contiguous incremental line outside of medulary bone.

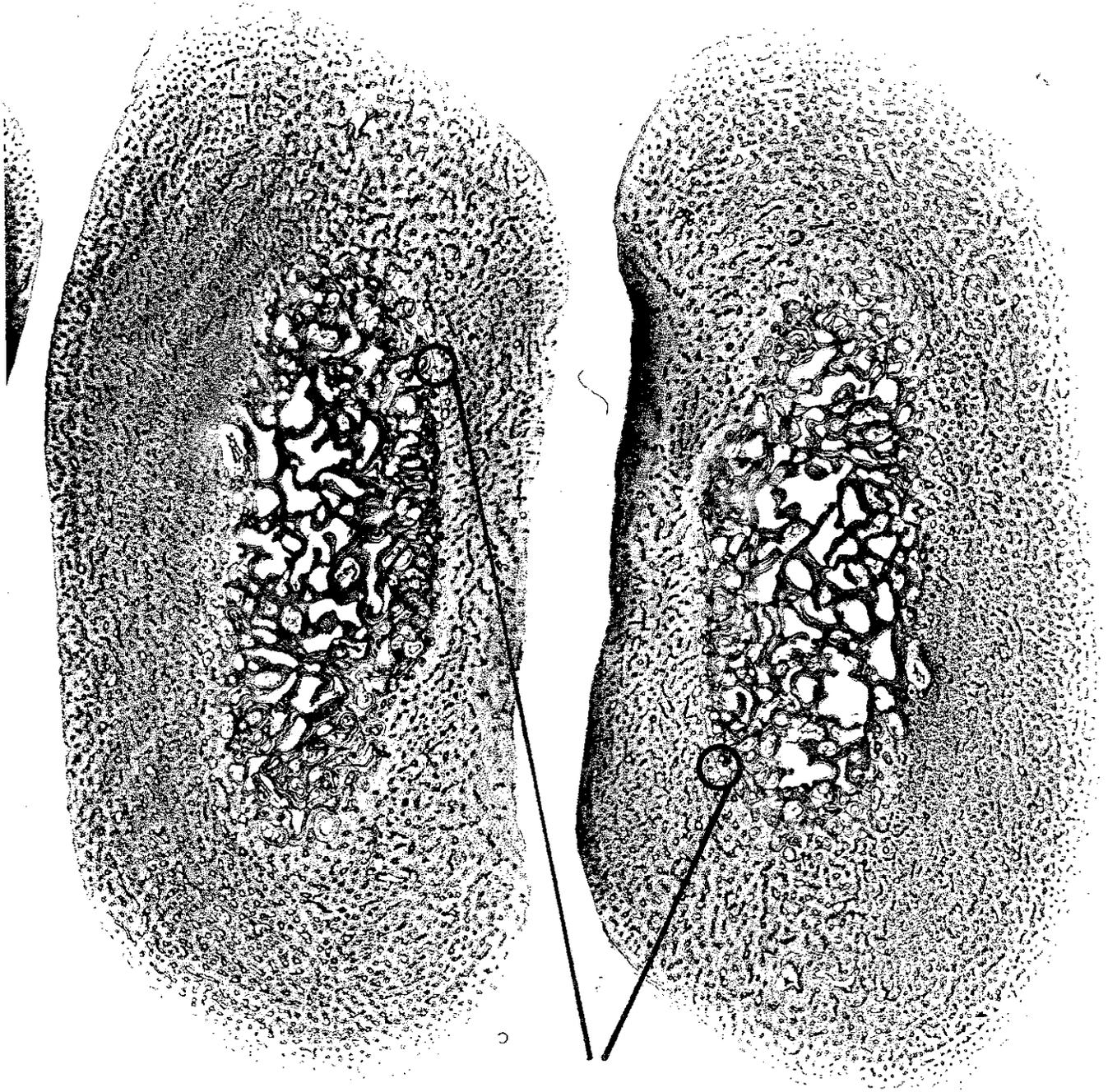


Figure 4

## Figure 5

Femur of 18-month old captive-reared Chelonia mydas (LC 201) showing evidence of three bands, (a, b, c) but only one clear incremental line (l).

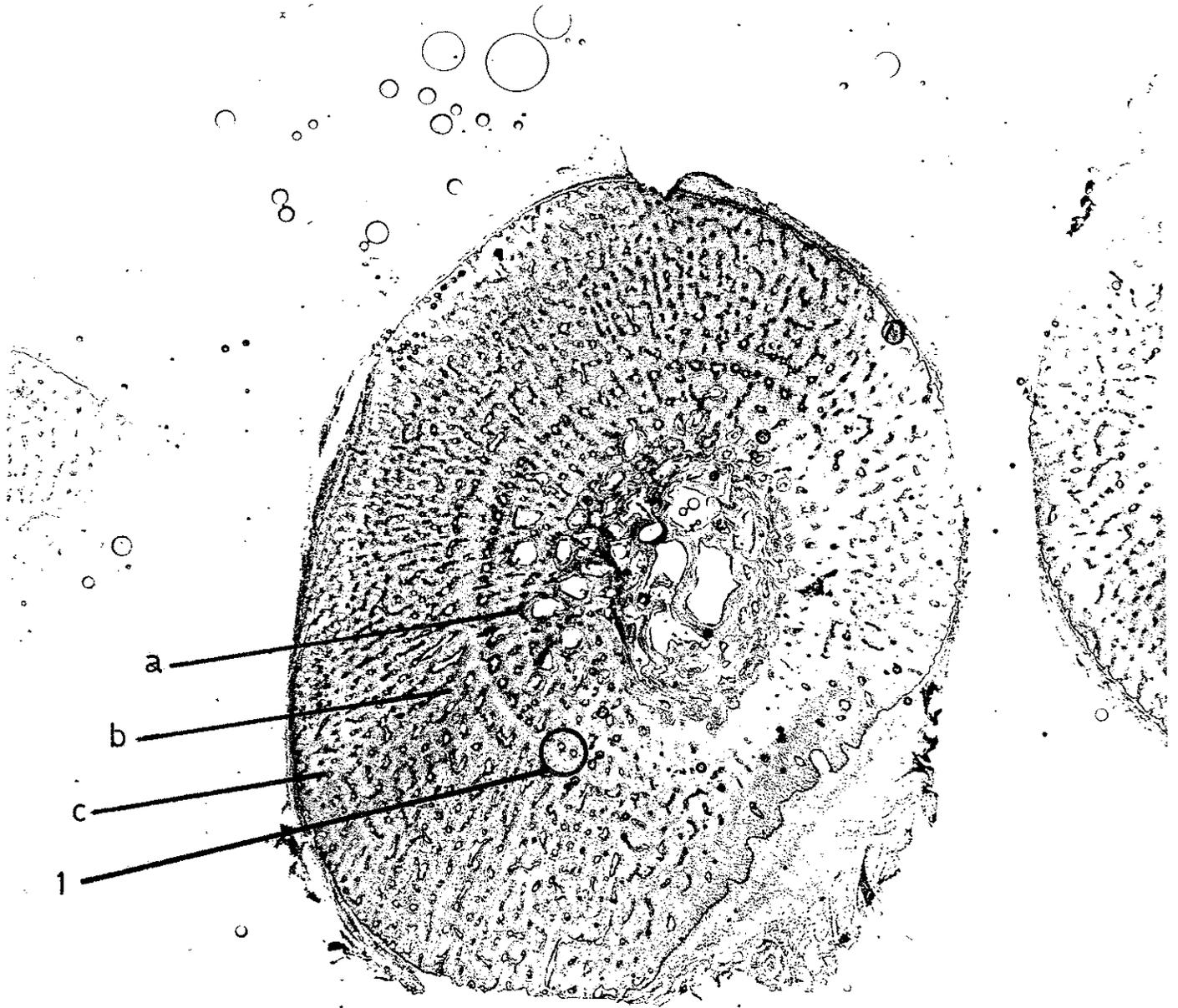


Fig 5

## Figure 6

Humerus of 18-month old captive-reared Chelonia mydas (LC 201) showing large (ventral) area of bundle bone (bb) two areas amplified in Figures 7 and 8 (F7 and F8)



Figure 6

## Figure 7

Humerus of 18-month old captive-reared Chelonia mydas (LC 201) showing ramification of lines in patch of dorsal bundle bone (1), and possible natal bone (2).



Fig 7

Figure 8

Humerus of 18-month old captive-reared Chelonia mydas (LC 201) showing many clear bands.

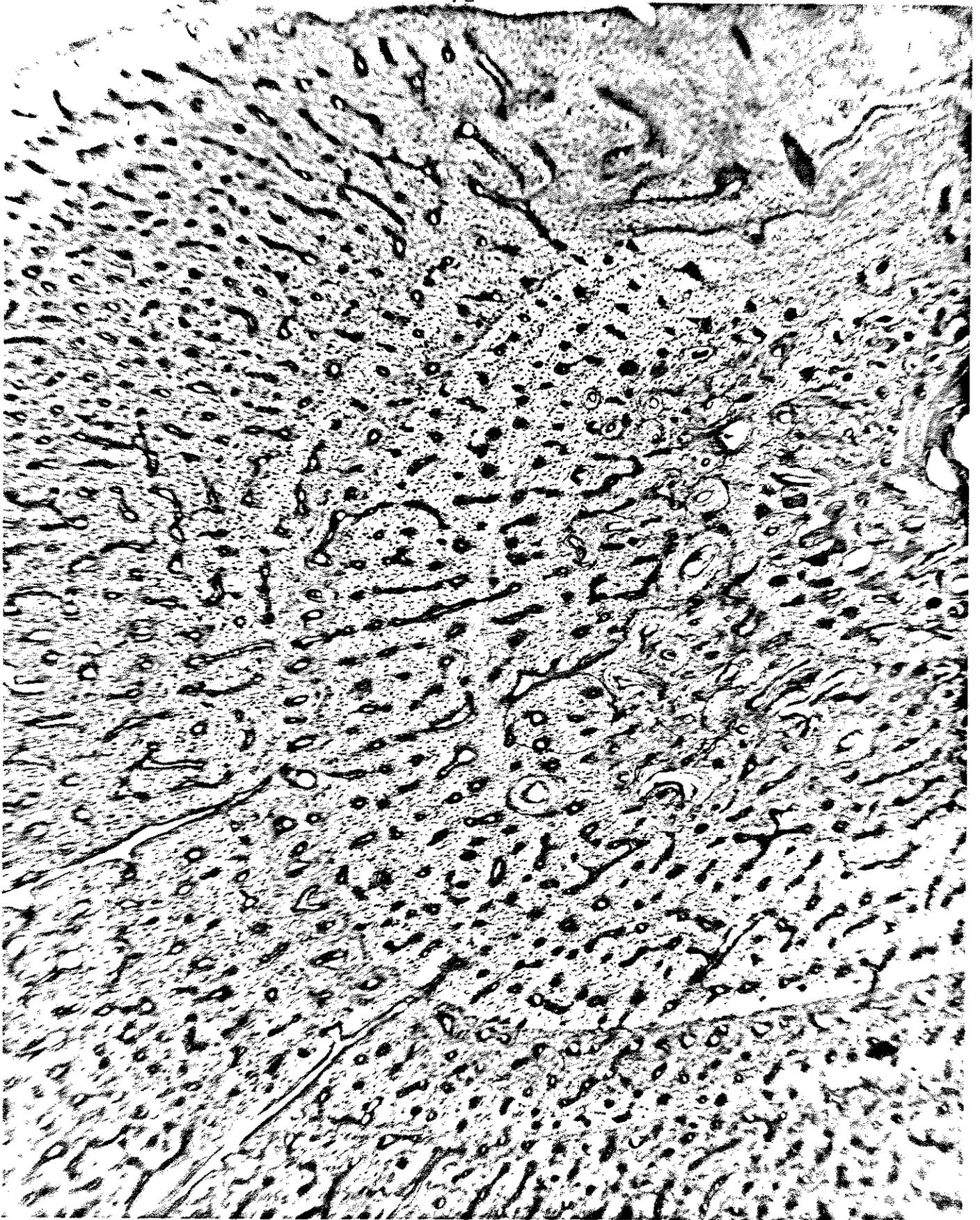


Figure 9

Humerus of 18-month old captive-reared Chelonia mydas (LC 201) showing  
less area of bundle bone and 5 distinct bands.

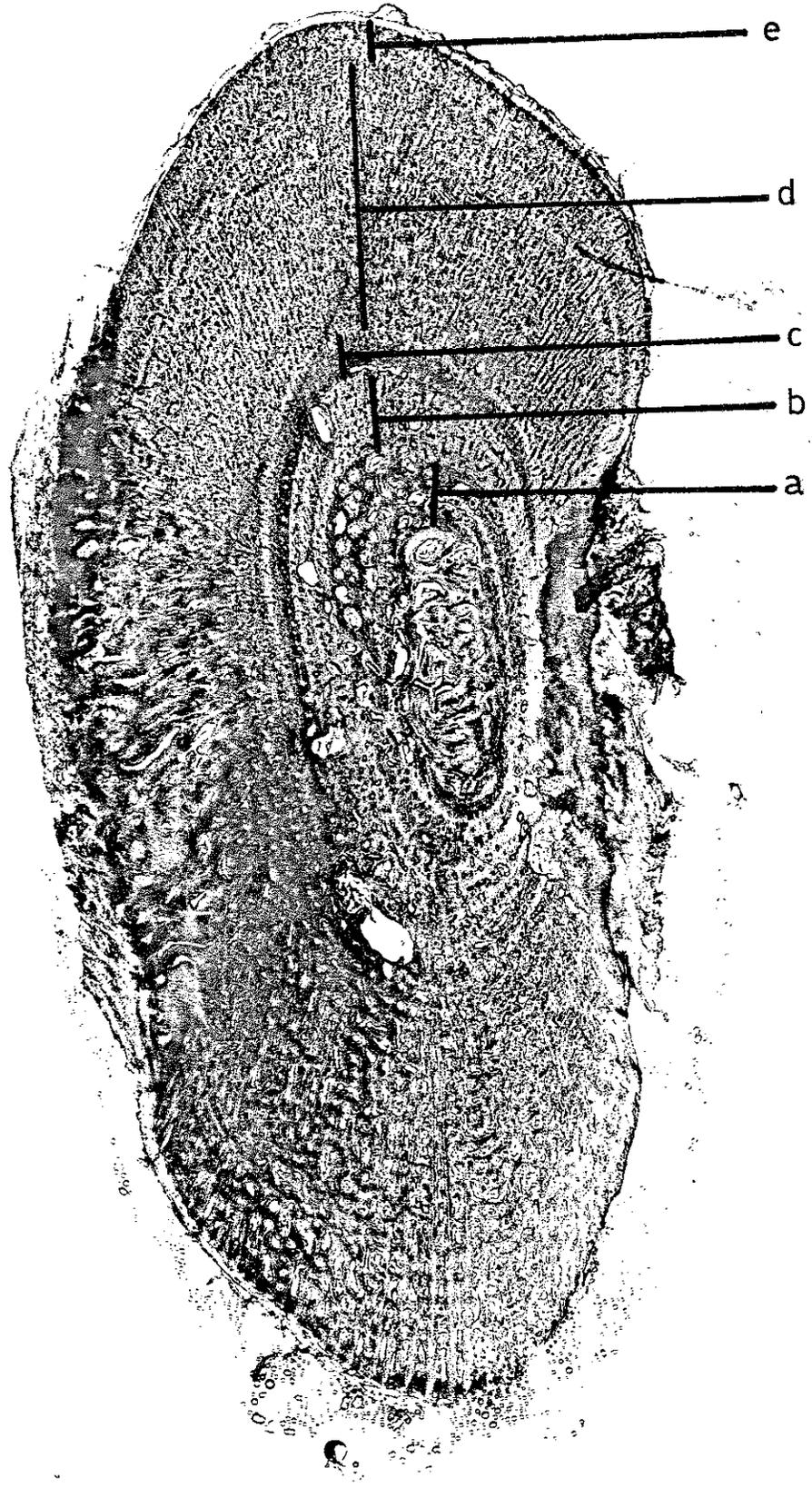


Figure 4

Figure 10

Humerus of 42-month old captive reared Chelonia mydas (LC 204) showing three bands separated by two concentric lines (1 and 2)

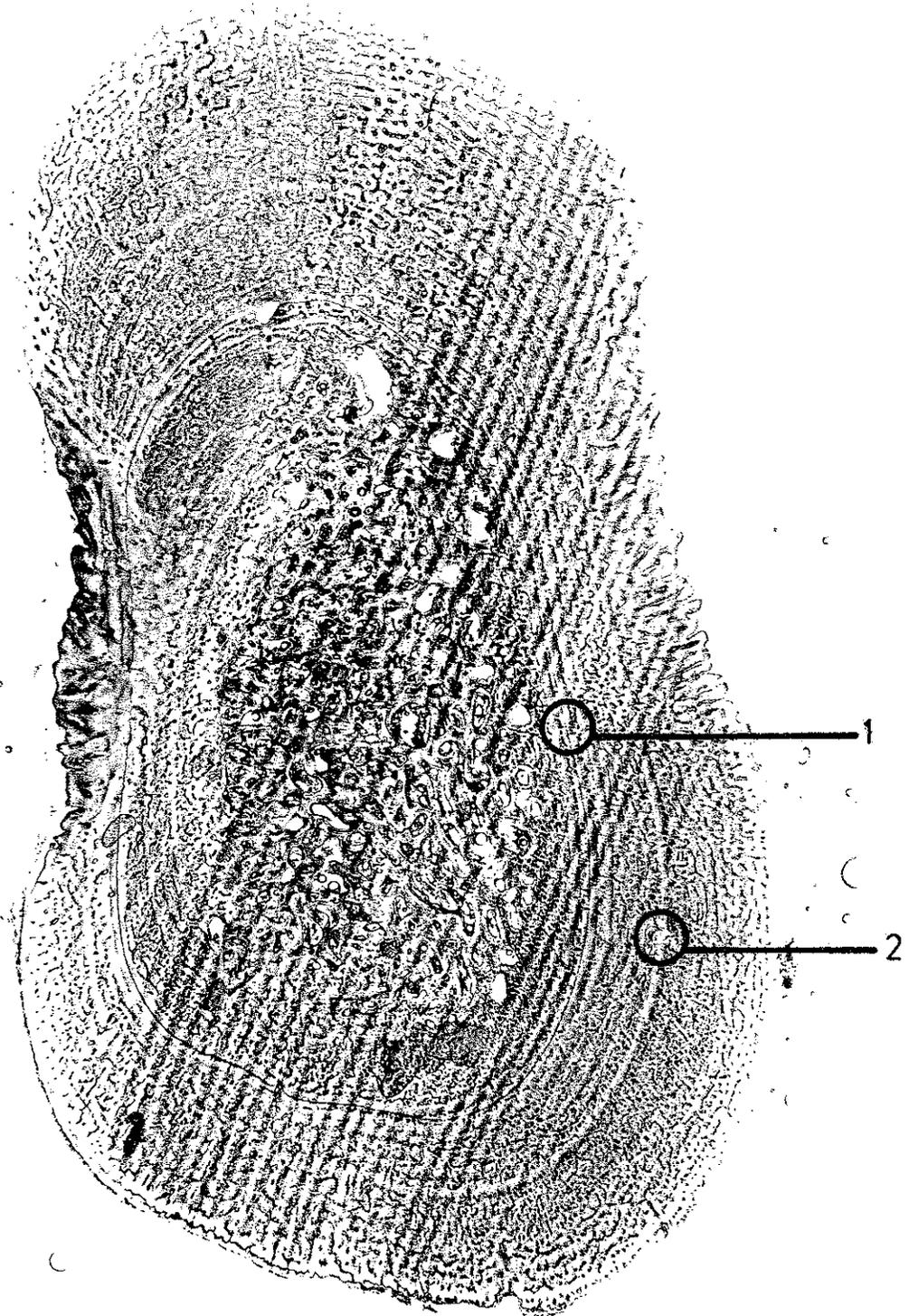


Figure 10

Figure 11

Femur of 42-month old captive-reared Chelonia mydas (LC 209) showing poorly defined bands and lines.



Figure 11

Figure 12

Femur of 42-month old captive-reared Chelonia mydas (LC 209) showing well defined lines (1).



Fig 12

Figure 13

Femur of 42-month old captive-reared Chelonia mydas (LC 209) showing one distinct incremental line (1).

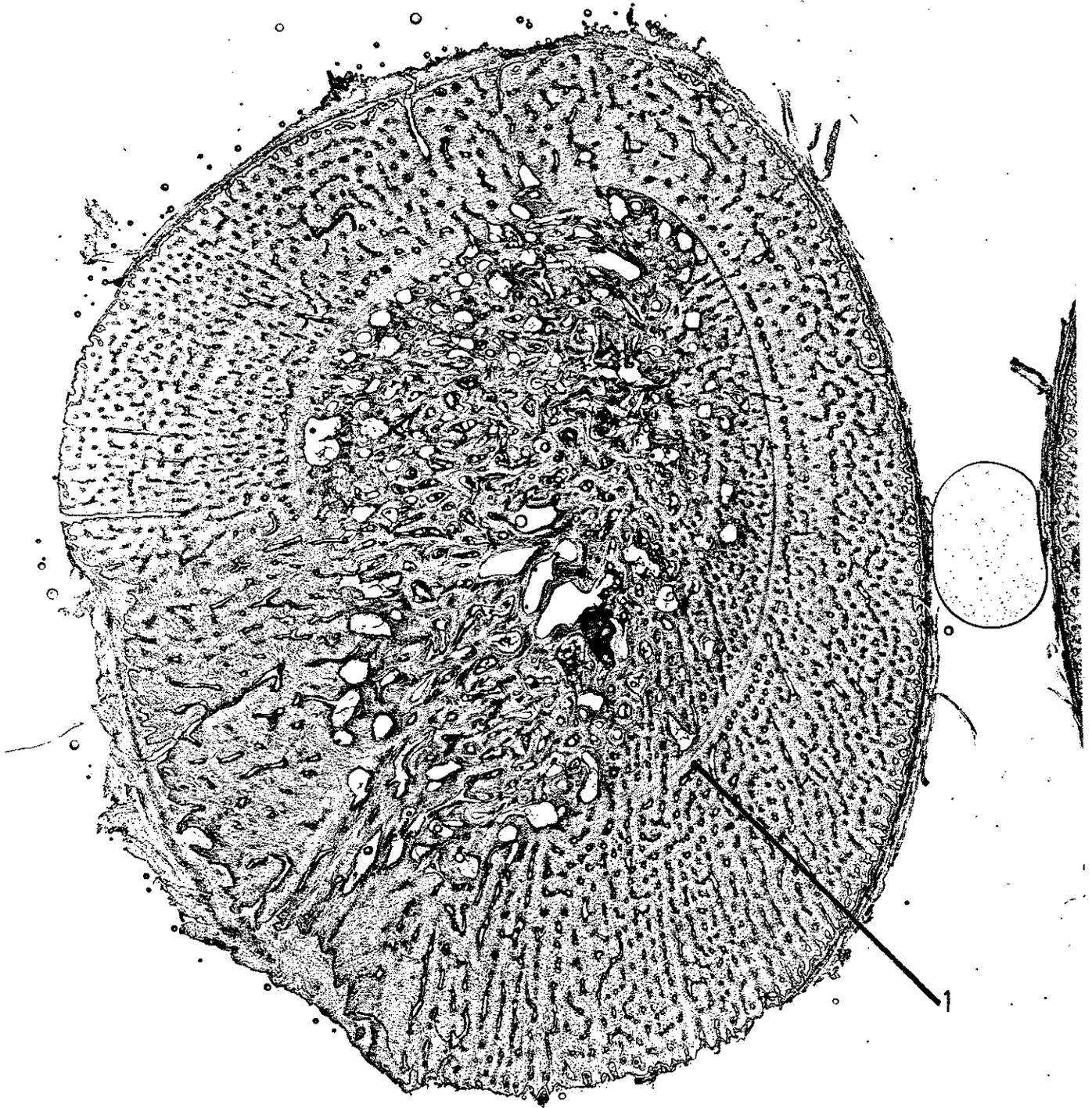


Figure 13

Figure 14 (a, b and c)

Humerus of adult Caretta caretta (USNM 881536) showing distinct incremental lines which fuse and ramify (A), disappear into spongy bone (B).

78'a

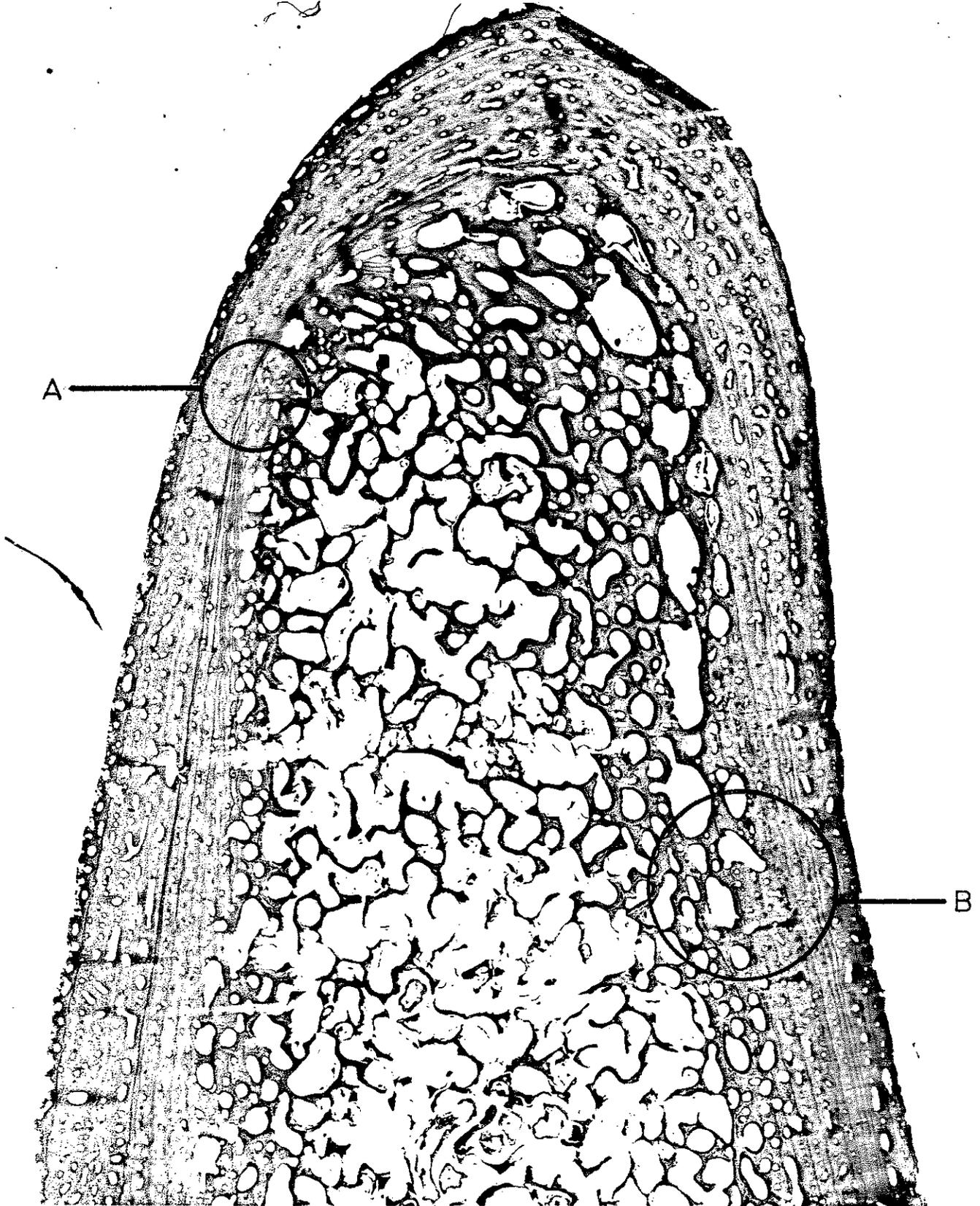


Fig. 14a

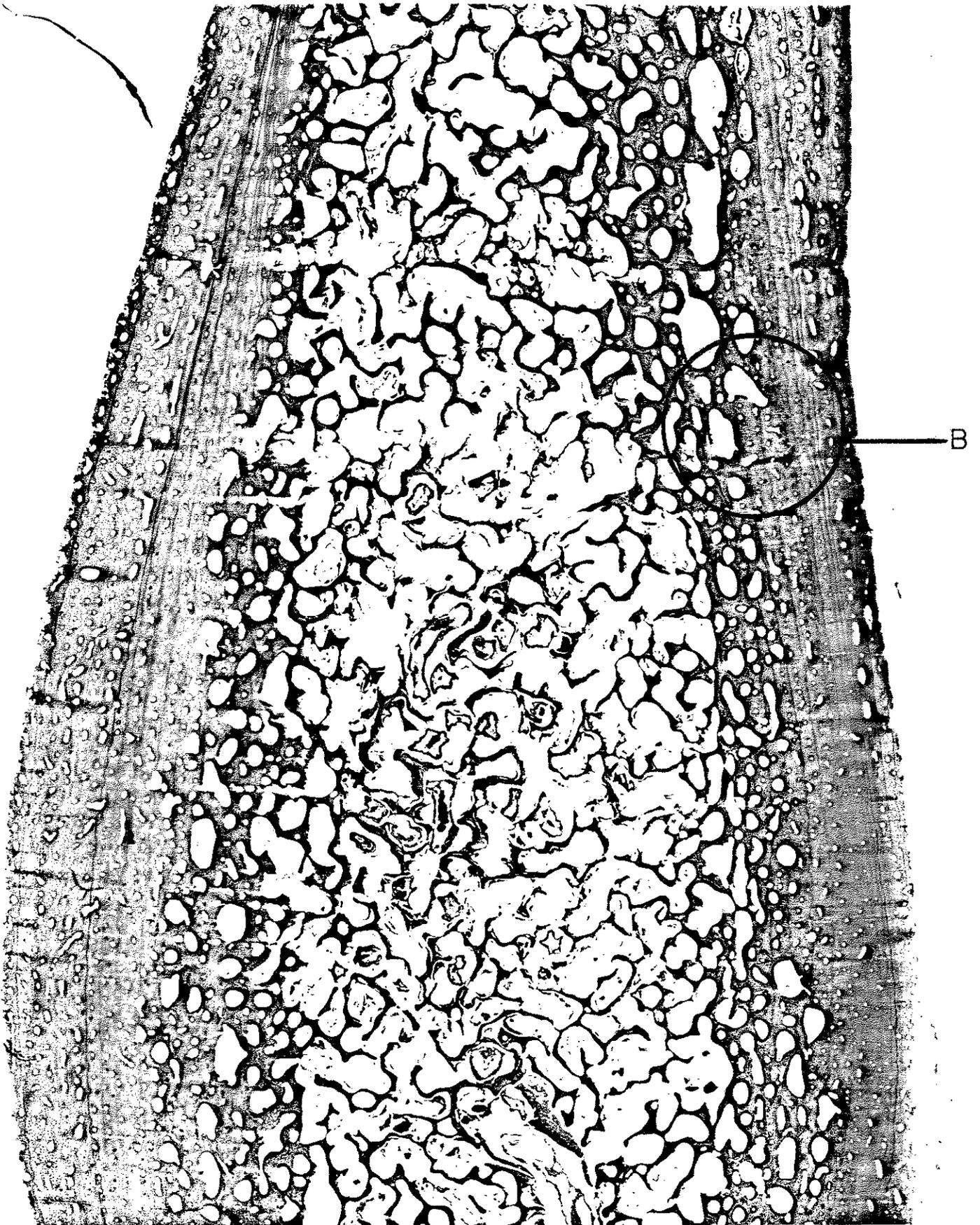


Fig 14 L

78'c

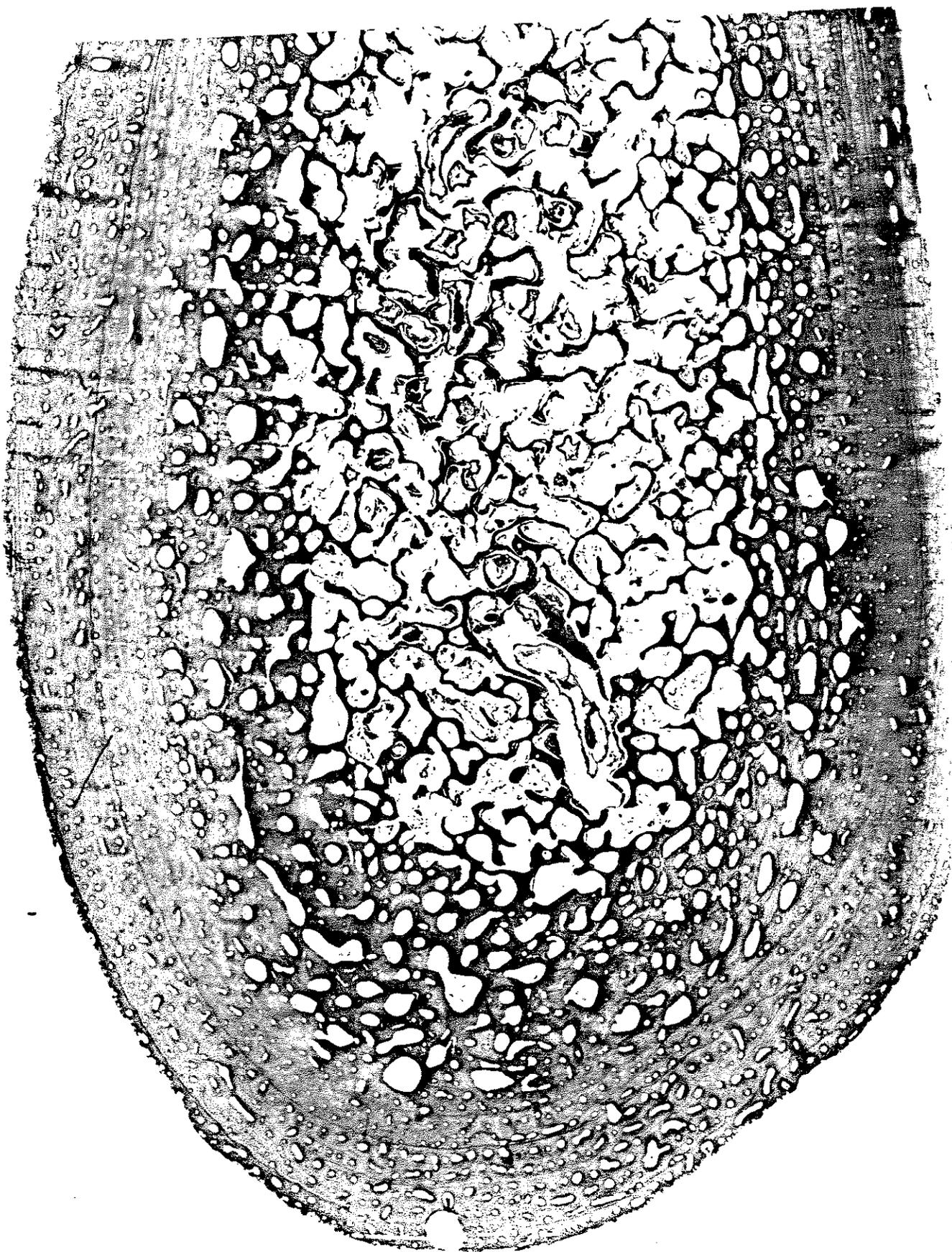


Fig 14 c

Figure 15

Humerus of adult Caretta caretta (USNM 881536) showing close up of area where lines fuse.

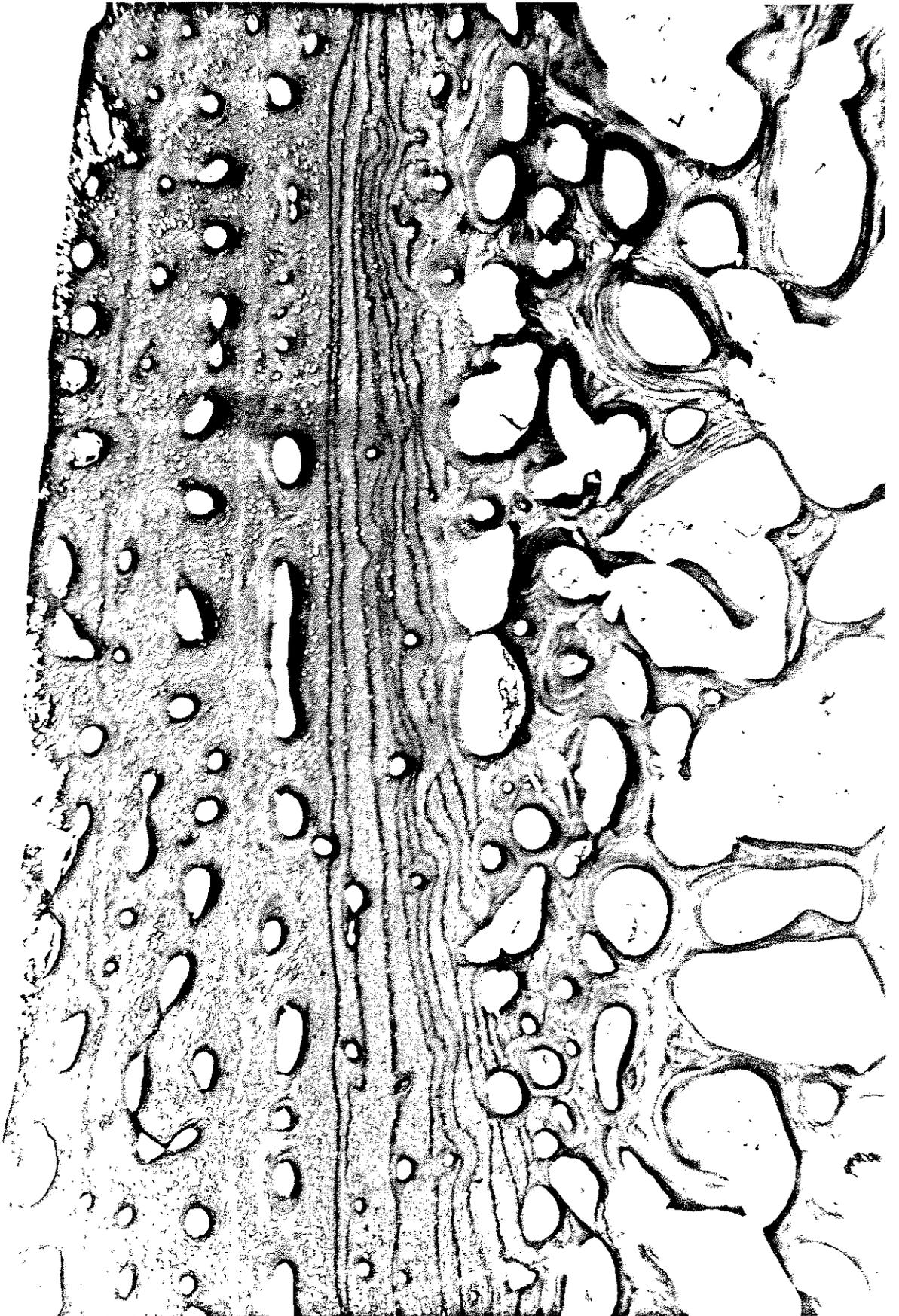


Fig 15

Figure 16

Humerus of adult Caretta caretta (USNM 881536) showing distinct incremental lines, some of which have an erratic, wandering form (W).

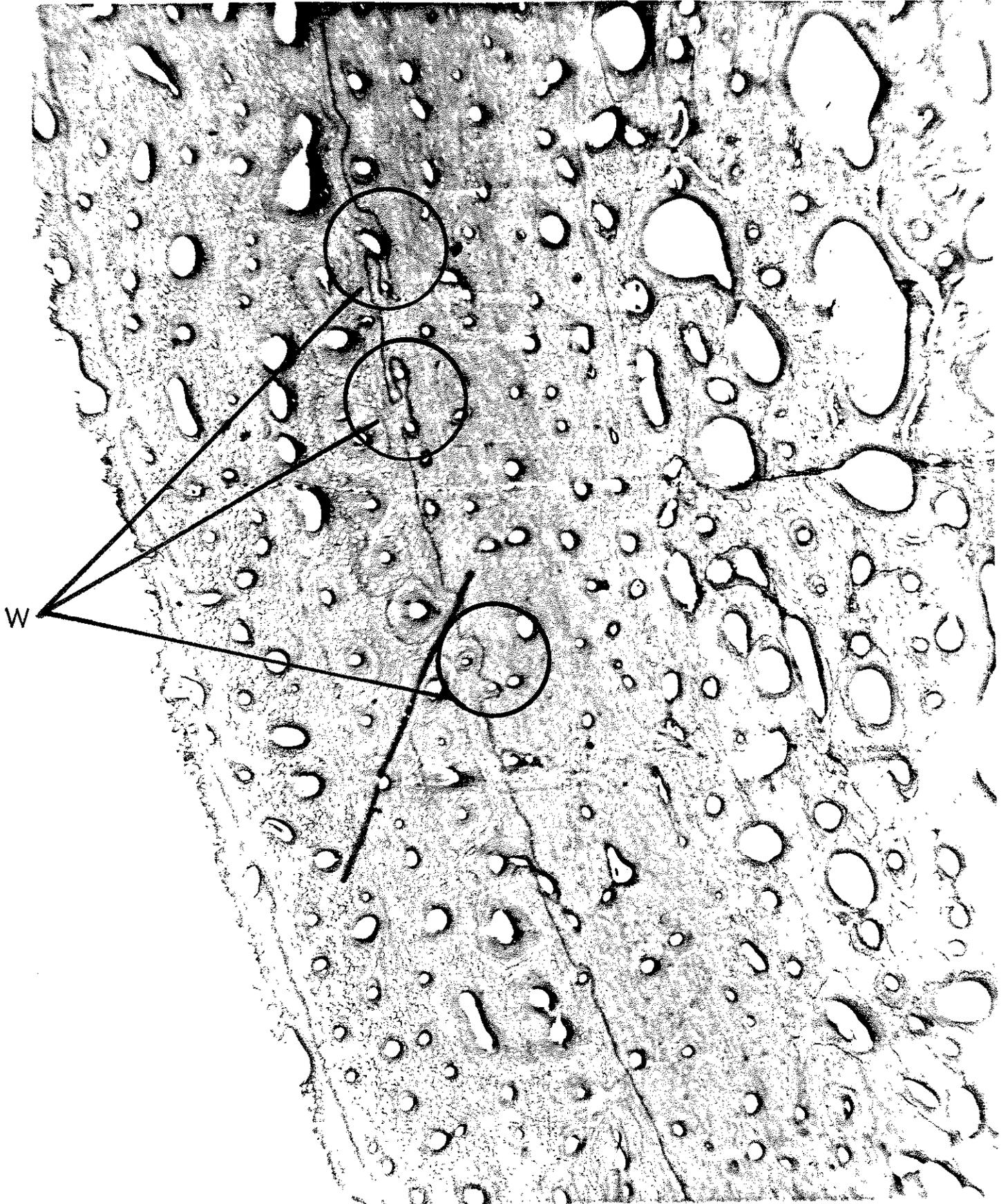


Fig 16

Figure 17

Microradiograph of an adult Eretmochelys imbricata (JFEi 151), showing banded pattern of mineral densities.

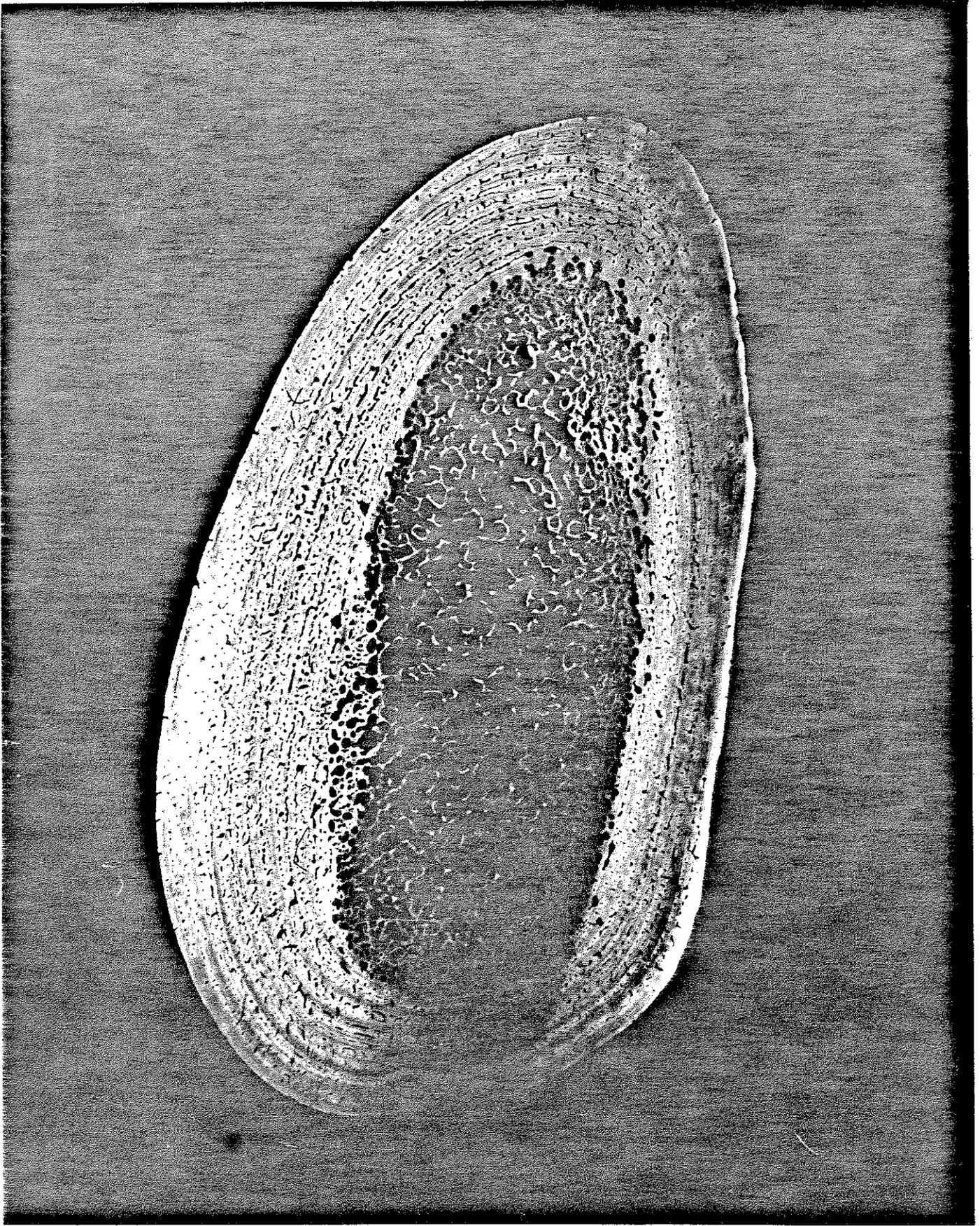


Fig 17

## EXTERNALLY VISIBLE GROWTH MARKS

Growth marks are visible on several external structures. The valves of many mollusks have obvious growth layers (Rhoads and Lutz, 1980). Most testudines have growth layers clearly visible on keratinous scutes of the carapace and plastron, and this provides an easy and valuable means of estimating age (Gibbons, 1976; Castanet and Cheylan, 1979). Sea turtles however, have smooth scales; evidently the outer (older) layers of keratin continually slough off. It is possible, especially in fast growing, young sea turtles to see conspicuous growth layers on scales of the carapace and plastron. However, in animals that are more than a year old it is rare to see more than 3 or 4 layers at the extreme edges of the scutes, showing that only a very short record of the most recent time period is clearly visible externally. The keratinous beak and claw also show banding, but like the scutes of the shell, only a few layers can ever be seen on an individual.

The exception to this is in the hawksbill turtle, Eretmochelys imbricata (L.). Scales of this species are thick and much valued and for millennian they have been traded for just this reason. On some specimens several dozen clear, but irregular, growth marks can be seen on scales of the carapace. Balazs (1977) has also remarked on this. However, not all individuals have numerous visible growth marks, and sloughing and abrasion still occur, although not as rapidly as in other sea turtles. Hence, this technique, still untested, will be of limited use.

While externally visible growth marks on scales are generally not usable for reliable estimates of age in sea turtles, it is possible that growth marks on the external surfaces of certain bones may provide a

means to estimate age without the need of laborious and costly sectioning techniques. Bryuzgin (1939) described growth marks on the os transversum and os supra angulare of 4 species of snake from Russia, and Petter-Rousseaux (1953) had similar findings with another snake species in France. Peabody (1958) described growth layers on the surface of several skull bones of a bullsnake in Kansas, including estopterygoid squamosal, quadrate process of the pterygoid, palatine and surangular. These layers were often consistent with layers seen in polished thin sections of other bones.

Johnson (1979) described a method for staining centra to accentuate growth marks, and this is a well-tried technique for aging teleost, as well as elasmobranch fishes (Jones and Geen, 1977). Minakani (1977) was able to find growth rings in centra of yellow-green Pitvipers, so the procedure deserves attention with reptiles studies. Pilot trials with marine turtle vertebrae have to date failed to show any clearly visible growth marks in centra. However, we are proceeding with this investigation.

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## X - RAY MICROANALYSIS

## DISCUSSION

The discovery that the growth layers and lines of arrest in sea turtles do not often show regular concentric circles, but fuse, separate, do loops, and fade makes it difficult to produce unequivocal counts from sections stained for conventional light microscopy. To better define the properties of the lines of arrest we used X-ray microanalysis. A thorough and recent discussion of this, and related, techniques is presented in Moreton (1981).

A similar technique has been used with success: Jones and Geen (1977) performed x-ray spectrometry analysis on vertebrae of spiny dogfish (Scualus acanthias). They scanned across centra and reported periodic ("cyclic") peaks in concentration of both calcium and phosphorus. Interpreted as evidence for annual cycles of metabolism of these two elements, the number of peaks and troughs in elemental concentrations were counted as a means to determinate age.

The technique is involved, but in brief, a specimen is exposed to an electron beam which causes characteristic x-rays to be produced, and these can be used to identify the elements bombarded. However, in this analysis elements with lower atomic numbers than Sodium (Na; i.e., 11) are not reliably detected.

Several wafers of bone were mounted on carbon stubs with aluminium tape and coated with carbon using a Denton High Vacuum Carbon Coater. These specimens were examined with a Philips 501B scanning Electron microscope and attached EDAX x-ray analyser.

A small portion of the bone's surface was scanned with the electron beam, and a line-spectrum profile was produced on a cathode-ray tube.

The surface was scanned until 400,000 x-rays were detected by the EDAX. These were analysed and plotted by a Data General Computer. Conspicuous peaks that could be clearly allocated to an element were obtained for only calcium (Ca) and phosphorus (P).

We then investigated the distribution of these two elements, in addition to Sulfur (S), on the surface of the cross section of bone by using x-ray mapping technique. The map of the specimen field consisted of 525 horizontal lines. For Ca and P each line was scanned and analysed for 1 second, for a total of nearly 9 minutes per specimen. For S each line was scanned for 4 seconds, for a total of 35 minutes per specimen. The map, a series of white dots on a black field, shows locations of a particular element in the path of the electron beam.

There was no discernable non-random pattern in any of the x-ray maps. However, these were mere pilots and further work will be needed.

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TETRACYCLINE LABELLING IN BONES OF SEA TURTLES

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## INTRODUCTION

In age determination studies, as in any scientific work, it is essential to calibrate the results. The examination of specimens of known age provides critical information that enables the interpretation of growth and age-related phenomena. Another valuable technique is to put known-time marks in "recording structures", hard tissues that grow by adding layers, including: bones, teeth, scales, otoliths, shells, and keratinous structures (see Klevezal', 1980).

There has been considerable interest in these in vivo marks for hundreds of years (Hoyte, 1960) with major symposia on the subject (American Journal of Physical Anthropology, 1968, Vol. 29, no. 2). Literally a spectrum of dyes and hundreds of metal salts have been administered to test their effectiveness as vital stains in bones and teeth, but the most common markers are alizarin red, lead acetate, and tetracyclines. However, each of these substances can affect the physiology of the animal, making the results difficult to interpret. Both alizarin red and tetracyclines can decrease or even arrest osteogenesis and dentinogenesis (Pizzolato and Lillie, 1968; Lillie and Fullmer, 1976). Tetracyclines, a family of at least seven similar molecules that are commercially available, are strong chelating agents and do have antimetabolic effects. However, at low dosages, i.e. short term labeling, the inhibitory effects are thought to be minor. Of the tetracyclines, oxytetracycline is considered the weakest metabolic inhibitor (Saxen, 1969).

In goldfish increasing mortality may result from increasingly larger dosages of tetracycline (Kobayashi, et al., 1964). However, Weber and Ridgeway (1967) reported no evidence of toxicity, increased mortality or

decreased growth even when salmon were fed several grams of tetracycline/kg body weight. Unless doses are very high, there is little chance of a deleterious effect during tetracycline labeling of vertebrate bones; low doses may even result in reduced mortality in salmon (Weber and Ridgeway, 1967).

The antibiotic effect of orally administered tetracyclines may disturb gut flora and fauna. Nevertheless, these compounds are relatively easier and safer to use and store than other substances used as vital markers (Harris, 1960; Saxen, 1969).

For this reason, following Milch's, et al's. (1957) discovery that bones of tetracycline treated animals have a band which fluoresces under UV light, these substances have been administered to a variety of organisms to mark recording structures and to thereby measure depositional rates. Previously other more toxic compounds, like lead-acetate, had been administered to animals to produce known time marks in recording structures (Okada and Mimura, 1938; Hiyama and Ichikawa, 1952). Yagi, et al., (1963) did one of the earliest experiments with tetracycline in age determination studies, marking the tooth of a fur seal. Since then various other studies have been made on a variety of animals, both vertebrate and invertebrate. The present study investigates the feasibility of labeling bones of sea turtles with tetracycline to later measure rates of bone deposition and interpret growth related phenomena.

## MATERIALS AND METHODS

The experimental animals were farm-reared green sea turtles, Chelonia mydas, L.), at the Cayman Turtle Farm (CTF) on Grand Cayman Island, British West Indies (see Ulrich and Parkes, 1978, for a description of this facility). Weighing between 15 and 31 kg, the experimental animals were 39 ( $\pm$  2) months of age. On 7 January 1982, six turtles (nos. 381 through 386) were injected with Liquamycin LA-200 (oxytetracycline; batch 06136) dorsal to the right hind limb in the area of the flexor tibialis externis. The dosages ranged from 4.9 to 27.9 mg oxytetracycline/kg body weight (Table 1). These six animals were transferred from a 29 m diameter stock tank to a 3 m tank and tagged. Samples were taken at three post treatment times: After 29 hours a wedge, about 5 x 15 mm, of marginal scute and peripheral bone was cut from the 10th right marginal of each turtle. Similar wedges were cut from two other control animals (CT 54 and CT 56) that came from the same stock tank, immediately after they were killed in the normal course of the CTF's activities. No sham (e.g., saline injected) controls were run in an attempt to minimize the interference to the normal running of the farm. On 26 May (139 days after treatment) turtle 384 was found dead, and a humerus, femur and wedge of marginal/peripheral were removed. On 29 September (265 days after treatment) wedges were cut from the remaining five live experimental animals. These samples are referred to as "29-hour", "139-day", and "265-day" samples.

Until it could be sectioned, each wedge was frozen in a plastic vial with a label and wrapped in aluminum foil or a dark bag. Wedges were mounted in paraffin and sectioned on an Isomet Low Speed Saw (Buehler, Ltd.) cutting 5 to 13 wafers per wedge. Each wafer was measured with a vernier

micrometer at each of the three angles of the triangle, to calculate an average thickness for each wafer; they varied from 0.15 to 1.65 mm in thickness. Sections were replaced in their vials and refrozen until they could be examined.

The wafers, without being fixed, decalcified, stained or permanently mounted, were laid on glass slides and examined under a Leitz Dialux 20 microscope with a Ploemopak mercury light source and H2 filter cube, which excites at a wavelength of 490 - 500 um. Photographs were made with a Vario Orthomat camera and Kodak Technical Pan Film 2415.

Each wafer was scanned and scored on the basis of fluorescence from 0 to 4: no glow, faint glow, definite line, bright line, brilliant line. Dorsal and ventral surfaces as well as the tip (Figure 1) were scored separately for each wafer. A series of five or six contiguous wafers for each wedge was chosen, maximizing the possible scores, for those wafers on either end of the wedge were often too thick or had too little bone to be read well. The scores for dorsal, ventral and tip were averaged for each wedge, and these three means were in turn averaged to give a grand mean for each wedge.

All five of the 265-day samples were examined for post treatment growth. From the distal tip of the peripheral bone, where dorsal and ventral surfaces meet, a distance of 3 mm was traced medially, first along the dorsal surface, then along the ventral surface. At each of these two points for each wafer the distance between the fluorescent line and the inner edge of keratin was measured. An average for each specimen was calculated for both dorsal and ventral surfaces. These values were then averaged for an overall mean for each specimen.

## RESULTS

Areas of yellow-green fluorescence were seen in wafers of both controls and experimentals, indicating the presence of auto- and/or low level dispersed fluorescence. This was especially notable in osteocyte lacunae and in keratin (Figure 1). Diffuse fluorescence was evident in many of the wafers to varying degrees of brightness and did not appear to be dosage-dependent. A line of fluorescence was seen at the outer edge of the bone, immediately interior to the periosteum, in an area inferred to have active osteogenesis. Occasionally diffuse and auto fluorescence made this line difficult to detect, but at least segments of lines were usually distinct in the treated animals (Figure 1), and normally they were more golden in color.

In the 29-hour samples the ventral surface consistently (except in controls) showed stronger fluorescence than the dorsal, and it was usually also brighter than the tip (Table 1). There is a strong relationship between fluorescence score and dosage (Table 1, Figure 2). Experimental animals with dosages of about 5 mg/kg body weight show little consist difference from the controls. Progressively greater doses from 10 to 20 to 30 mg/kg body weight result in progressively brighter fluorescent lines.

The 139-day sample showed no fluorescence in peripheral bone, humerus, or femur. In contrast, the 265-day samples all had distinct fluorescent lines (Figure 3). However, the dorsal surface usually glowed brighter than either the ventral surface or the tip. In general, the average score for each specimen was comparable to its 29-hour post treatment score (Table 1, Figure 2).

There was no simple relationship between wafer thickness and

fluorescent score. As long as the wavers were between 0.15 and 1.65 mm thick, the mark could be readily seen.

There was no indication of any relationship between post-treatment bone deposition and tetracycline dosage level for either dorsal or ventral surfaces, or for the overall average (respective  $r$  values = -0.49, 0.13, and -0.18; d.f. = 3). Dorsal growth was generally less than ventral; in four turtles the dorsal value was between 0.5 and 0.8 of the ventral, and in one specimen it was 1.4 times the ventral.

## DISCUSSION

These results are comparable with previously reported findings; however, the majority of tetracycline labeling has been done with mammals, and no such studies appear to have been done on turtles. Indeed, there is little information on general curative doses for reptiles, yet considerable difference between the few values that have been published: Fry (1973: 140; 1977: 790) recommended daily doses of 6-10 mg oxytetracycline/kg, but Marcus (1981: 93) suggested two doses per day of 50 mg tetracycline/kg.

Factors likely to affect the mark include: the drug used, dosages, route of administration, species, age and sex of the animal, as well as the storage and preparation techniques. Saxen (1969) discussed the effects of different tetracyclines, concluding that in order of increasing intensity of fluorescence they are: oxytetracycline, chlorotetracycline, methacycline, tetracycline, and dimethylchlorotetracycline. This ranking also applies to the strength of the effects these drugs have on metabolism.

The intensity of fluorescence is related to tetracycline dosage level in: goldfish otoliths (Kobayashi, et al., 1964), salmon hard tissues (Weber and Ridgeway, 1967) and human bones (Frost, et al., 1960). The present findings are consistent with these.

The route of administration has no obvious importance; tetracyclines were effective in marking bones of rats and rabbits regardless whether administered intramuscularly, intraperitoneally, intravenously, subcutaneously, or orally (Milch, et al., 1958). Hence, little importance has been paid to this variable in recent studies. However, oral doses may not only be difficult to administer but may have adverse effects on gut symbionts. The injections used in this study had no obvious effect on the animals.

There is little information comparing the effect of species. Rodent

(and rabbit ?) bones showed twice as much fluorescent brightness as did those of dogs or humans with equal dosage levels. Age differences in both intensity and location were noted, and related mainly to patterns of active osteogenesis. There was no evidence of a sex related difference in these tetracycline treated animals (Milch, et al., 1958).

The delay in fluorescence is minimal; in rodents it is evident within 20 s of drug administration. Tetracycline induced fluorescence occurs not only in bone and other hard tissues, but also in soft tissues, although the effect persists only in hard tissue (Milch, et al., 1958). In salmon, Weber and Ridgeway (1967) claimed that deposition of fluorescent layers was immediate. The present results show that an effect can be detected in sea turtles within 29 hours of treatment.

Harris, et al., (1962) described in detail both diffuse and auto fluorescence in studies with tetracycline treated dogs. They, like Milch, et al. (1957) and others, also emphasized that all actively growing areas of bone are labeled. Although diffuse, nonspecific fluorescence was conspicuous in the present study, it was generally less intense than fluorescence at the line of active bone growth, and it generally took on a different color, less green, color.

Persistence of the fluorescence was of considerable concern to early workers. Milch, et al. (1958) felt it important to report that the labels were visible for six months in rabbits. They concluded that studies were needed to determine the half life of the bone fluorophor, but little attention has been paid to this problem. Frost (1961), however, was able to report that the tetracycline label lasted for at least nine years in a human clavicle, unless the bone was removed by resorption. From other published reports (see Domning and Myrick, 1980; Gurevich,

et. al., 1980), there is little question that the label is long-lasting, although Frost, et al. (1960) reported a fading of label after six years in living human bone.

Frost, et al. (1960) observed no evidence for diffusion of fluorophor out of the originally labeled mark in human bone that had been sectioned and mounted for three years. The present study shows the same fixity of label. Numerous workers on cetaceans have specimens at hand that bear on the problem of permanence of label after long periods of storage, but few have paid attention to this (Hohn, pers. com.).

Another facet of this problem that has been either taken for granted or ignored by recent workers involves the way specimens are treated and prepared. It is commonly claimed that the fluorescent mark is lost after decalcification, and for this reason it is often reported that specimens were not preserved in formaldehyde because the weak formic acid that forms acts as a decalcifying agent. In many studies tissues are preserved by freezing. Seventy percent ethanol has also been recommended as a preservative that will not effect the mark, but Frost, et al. (1960) stated that there is a loss of fluorescence "over many weeks". However, there seem to have been no clear studies conducted to test these techniques (see Frost, 1969). Freezing seems an acceptable, if inconvenient, storage technique.

Weber and Ridgeway (1967) were concerned also with the photolytic effect of normal solar illumination on the tetracycline induced fluorophor. Scales of salmon in outside ponds lost the label in two or three days whereas scales of fish in inside tanks, protected from sunlight, showed fluorescence at least 13 months after treatment. If in fact the photolytic effect is so great, one might expect the label on the dorsal surfaces of the turtles'

peripheral bones to be consistently duller and less distinctive than the label on the ventral surfaces, which were protected from direct sunlight. This is true for the 29-hour samples, but the converse applies to the 265-day samples. It is relevant however, that in sample 381 the bright dorsal line is interrupted where the pigment in the shell above it is light rather than dark. Thus, it is possible that the fluorophor under the dorsal, but dark-shelled, surface is better protected than that on the ventral, but light-shelled surface, but there is no simple explanation for reversals of relative brightness in dorsal and ventral fluorescent surfaces.

There is no ready explanation for the absence of fluorescence in the 139-day sample of no. 384. The bones were thawed and possibly heated in the summer sun while in transit, and perhaps this destroyed the fluorophor.

Dosage has no apparent effect on growth when it is between 5 and 30 mg oxytetracycline/kg body weight.

Wedge thickness does not seem to be critical. Frost (1961) recommended that sections be 40  $\mu\text{m}$  thick, and Harris, et al. (1962) cut sections at 100  $\mu\text{m}$ , but in the present study wafers as thick as 1650  $\mu\text{m}$  showed clearly visible fluorescent marks.

## CONCLUSIONS

Oxytetracycline, when administered intramuscularly in doses of 5 to 30 mg/kg body weight, results in fluorescent lines in the periosteal region of bones of captive green sea turtles. Brighter fluorescence is found with stronger doses. The label is evidently laid down very rapidly and can be detected in peripheral bones within 29 hours, when the ventral surfaces of the bone show brighter fluorescence than do the dorsal. After nearly nine months fluorescent lines are present, with about the same level of conspicuousness as was evident right after treatment; however, those on the dorsal surface are brighter than those on the ventral, a reversal of the previous condition. As well as brightness of the label, osteogenesis also appears to be related to dosage level, but in an inverse manner.

This represents the first study of bone labeling in marine turtles, so the results can only be compared to findings on other animals, especially mammals. In general the results are consistent with those of earlier studies, however, the careful observations made by early workers are not often repeated in more recent research, and it is not always possible to make comparisons because insufficient information is reported: e.g., Nielsen (1972) in a much cited and valuable paper, reports neither the drug nor the dosage used in marking.

Future work will examine the effects of other labels and the effects of different preservation techniques. Nonetheless, a technique is now available for quantifying the deposition rates of turtle bones, and applying this toward age determination studies. It should be possible not only to mark captive animals, but also to mark wild sea turtles.

The best label will be conspicuous yet have minimal effect on the

animal. The results of this study indicate that dosage levels of about 30 mg/kg body weight will be suitable. It may be possible to use potentiators, substances which act synergistically with tetracycline to enhance fluorescence. This was described for salmon and has also been used in birds and mammals (Weber and Ridgeway, 1967), but the technique has not been used recently.

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Table 1. Body weights, dosages and fluorescence scores of Chelonia mydas (L.) injected with Liqumycin LA-200.

Specimen N <sup>o</sup>	Date 1982	Body weight (kg)	ml. LA-200 injected	Dosage mg/kg	Average fluorescence score			Grand Average
					Dorsal	Ventral	Tip	
CT 54	7 Jan.	15.4	0	0	0.50	0.50	0.33	0.44
CT 56	"	25.4	0	0	0.17	0.00	0.67	0.28
381	"	24.5	0.6	4.9	0.83	1.67	0.67	1.06
382	"	24.9	1.2	9.6	1.67	1.83	1.67	1.72
383	"	16.3	1.6	19.6	2.00	3.00	1.67	2.22
384	"	19.1	0.5	5.2	0.00	0.50	0.83	0.44
385	"	21.8	1.1	10.1	2.00	2.50	2.00	2.17
386	"	22.2	3.1	27.9	2.33	3.83	3.33	3.17
381	29 Sept.				1.75	1.88	0.50	1.38
382	"				2.80	0.40	1.80	1.67
383	"				2.80	2.00	0.00	1.60
384	26 May.				0.00	0.00	0.00	0.00
385	29 Sept.				2.75	2.00	2.17	2.31
386	"				3.83	2.33	3.58	3.25

## FIGURE LEGENDS

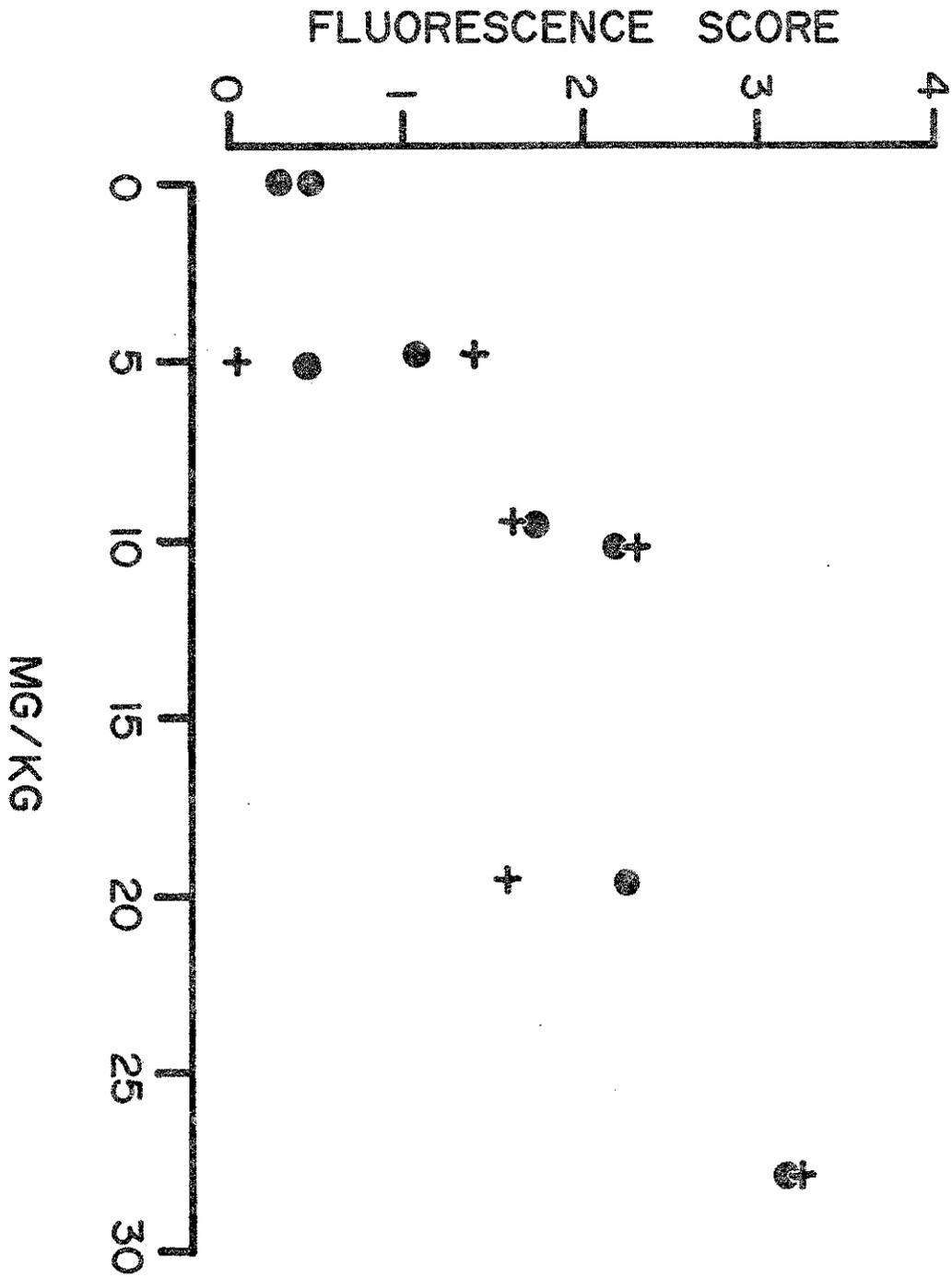
- Figure 1. Photomicrograph of wafers of periferal bone of captive Chelonia mydas (L.).  
(A) Control.  
(B) Animal dosed with 10 mg oxytetracycline/kg body weight:  
D = dorsal surface, V = ventral surface, T = tip.

- 110  
Figure 2. Relationship between fluorescence score and dosage of oxytetracycline in captive Chelonia mydas (L.);  
0 = 29 hours sample; + = 139 day and 265 day samples.

- 111  
Figure 3. Photomicrograph of a wafer of periferal bone of captive Chelonia mydas (L.); dosed with 28 mg oxytetracycline/kg body weight:  
D = dorsal surface, V = ventral surface, T = tip.









## THE EYE LENS AS A TOOL FOR AGE DETERMINATION

Background

The vertebrate eye lens shows numerous age related changes: its gross volume and weight (both wet and dry) increase; the relative amounts of insoluble, or aggregated, proteins increase; the relative amount of alpha and beta crystallines increase while other crystallines decrease; the relative amount of dextrorotatory amino acids increase; the relative amount of sugar increases; and the activities of several enzymes change. An understanding of the morphology and ontogeny of this organ helps in understanding the first relationship:

An invagination of thickened ectoderm, "lens placode", forms the "lens vesicle". The cells that make up this structure grow and occupy the central space, but a layer of unmodified cuboidal cells, the "lens epithelium", remains to cover the original anterior surface of the vesicle. The lens vesicle secretes a lens capsule which entirely envelops the lens. Were the half dome of lens epithelium ends, its cuboidal cells give rise to cells that elongate within the lens to form fibers; the fibers continue to grow until each essentially forms a semicircle, with the ends contacting other fibers at the "suture plane". Although lens growth is most rapid before maturity, fibers are generated and growing throughout the life of an individual, and the lens takes on a form much like an onion with innumerable enveloping layers. As fibers are gradually pushed away from the epithelium they are cut off from the external supply of oxygen, and they then harden (sclerose) and finally die. At the center of these layers of fibers is the "embryonic nucleus", with the oldest and hardest fibers of all. "The lens is thus unique

among the organs of the body in that its development never ceases, while its senescence commences even before birth" (Walls, 1942:113).

The phenomenon of unending lens growth was reported as early as 1833 by the British physician Priestly Smith, working with human lenses. However, it was not until 1959 that Rexford D. Lord used this phenomenon as an interpretative tool to determine the ages of wild animals. He first worked with cottontail rabbits (Sylvilagus floridanus) and subsequently more than 25 species of mammals in 6 taxonomic orders have been studied. Birds are less extensively examined; only about 5 species in 2 orders have been investigated. A few fishes have also been studied (Table 1); however, age determination studies in fish can rely on more easily accessible or more deterministic organs such as scales and otoliths (or "sagittae", Pannella, 1980).

Evidently the eye lenses of two classes of vertebrates have never been investigated for age dependent changes. In his review of "Aging Phenomena in Reptiles", Gibbons (1976) did not make one mention of eye lenses; two bibliographic reviews on Age Determination of Wildlife, commissioned by the U.S. Department of Interior (Graff, 1981; Madsen, 1967), likewise showed an absence of these studies in Amphibia and Reptiles.

The majority of studies on gross changes of lens aging have dealt with oven dried weights of formalin preserved lenses because the early work reported that wet weights of preserved lenses were less strongly correlated to age (Lord, 1959). Few studies of age determination have examined fresh weights, because it is necessary to have access to a balance sensitive to 0.01 mg within a few hours of the animal's death.

Coupled with the increase in gross weight are several age dependent molecular changes. In most vertebrate lenses at least 50% of the mass is water, and the majority of the remainder is protein, although sugar and enzymes are present. Age related changes are reported in all of these classes of molecules.

The fiber cells of a lens are singularly long, flexible, stable and devoid of normal cytoplasmic inclusions, hence they are rich in proteins. The lens proteins can be grouped into three general categories: water soluble-low molecular weight; colloidal-intermediate molecular weight; and water insoluble("albuminoid")-high molecular weight. With increasing age, masses of each of these categories increase, however, the total weight of large molecules show a relatively greater increment. In dogfish (Mustelus canis) the insoluble/soluble ratio increases from 1:5 to 2:1 with the change from youth to maturity. (Zigman and Yulo, 1979). Similar age-dependent changes have been reported from the lenses of other vertebrates (eg. humans, Zigman et al., 1976). Because the insoluble proteins are approximately twice as heavy as the soluble, it has been assumed that protein aggregation of the smaller units results in the relative increase in quantity of the larger proteins (Zigman and Yulo, 1978; 1979).

The majority of these protein molecules are in the form of crystallins, of which four have been identified and named "alpha" through "delta". Delta crystalline, known only from birds and reptiles, comprises more than half of all crystalline in a lens, but it is the first crystalline to be formed in the embryonic eye, and its synthesis stops soon after hatching. Thus, the relative amount of delta crystalline

decreases with age. On top of this, there is an age related reduction in the amount of alpha-helix in delta crystalline (see review in Williams, et al., in press). Age related changes have not been studied in turtle crystalline, but general characteristics of delta crystalline have been described for red-eared sliders (Pseudemys scripta) (Williams, et al., in press) and green sea turtles (Chelonia mydas) (De Jong, et al., 1980).

In addition to different classes of crystallines, there are distinct species of molecule within each crystalline. For example, three size ranges of macromolecules have been described for bovine alpha crystalline with molecular weights of :  $6$  to  $9 \times 10^5$ ;  $0.9$  to  $4 \times 10^6$ ; and  $1 \times 10^7$ . With aging there is an increase in relative amount of the larger macromolecules: "there appears to be a transformation of lower molecular weight alpha crystalline to higher molecular weight species ... neither amino acid composition nor subunit size can be involved to explain the striking change in macromolecular size" (Spector, et al., 1971; 681). These authors reported that together with the age related increase in alpha crystalline macromolecular aggregation there is an increase in the amount of glucose present, and the availability of this sugar is related to the transformation of larger macromolecules.

The crystalline composition of a lens seems to be regulated by the development of the lens, not its absolute age. Brahma (1980) reported that normal and regenerated lenses of African Clawed frogs (Xenopus laevis) showed identical bands for crystallins in isoelectric focusing.

In addition to age dependent changes in molecular composition, there are changes in molecular structure of "innert proteins" (i.e. those that

are unchanged by normal metabolism after they have been synthesized). Animals synthesize only one isomer of amino acids, the levorotatory form ("L-enantiomers" or "L"), and these automatically change to the dextrorotatory form ("D") as the population of molecules moves towards equilibrium of isomers. The rate of this change, or "racemization", is dependent on pH, temperature and the amino acid involved. Aspartic acid is a convenient molecule to examine because its racemization half life is shorter than in other amino acids.

Bada, et al. (1980), and Masters, et al. (1977; 1978) have reported age related increases in D/L ratios of aspartic acid from lens nuclei of humans, gorillas, zebras, sea lions and dolphins. With sufficient information on a population's racemization rate it is possible to calculate the age of an individual given its D/L value.

As mentioned above, Spector, et al., (1971) reported an age dependent increase of glucose in bovine lenses, and this is related to an age dependent increase in macromolecule aggregation. Enzymes active in glycogen metabolism show a small decrease in activity in aging of the low beta fraction, a crystalline which shows little age related change. Other enzymes that are part of the more age dependent alpha crystalline fraction may have more age related changes (Orloff, et al., 1976).

Endopeptidases show an age related increase in activity in the bovine lens. As with many of these functions, the curve accelerates rapidly at first (to about 5 years of age) then rises less steeply (Hockwin, et al., 1976).

Discussion

As in any study of age related changes, the first problem is to solve is acquiring know-age samples. Fortunately a series of nearly 100 lenses have now been collected from Cayman Turtle Farm, and these represent know-age specimens from 2 weeks to 14 years of age.

Fresh weights were recorded and these have been partially analysed (see attached draft "Eye lens weight as an indicator of age in captive green sea turtles"). The final analysis with a series of lenses collected at the end of September 1982 is in preparation.

The results show an age dependent increase in weight which accelerates rapidly at first and then appears to be asymptotic at about 10 years of age. This is evidently the first time any such analysis has been done for any reptile, but the results are consistent with those of other studies on mammals and birds.

This indicates that other age-related phenomena reported in vertebrate eye lenses apply to marine turtles, and additional analyses are being done in cooperation with other laboratories: Dr. S. Zigman of the University of Rochester is analysing a series of lenses for aggregated proteins; Dr. I. Piatagorsky of the National Eye Institute is analysing several lenses for crystalline composition; possibly Drs. Bada and Masters of the Scripps Institute of Oceanography will be able to analyze several lenses for racemization ratios. Because these analyses are being done at various other laboratories which cannot do this work as first priority, it may be some months before results are obtained.

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## EYE LENS WEIGHT AS AN INDICATOR OF AGE IN THE CAPTIVE

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## INTRODUCTION

Vertebrate eye lenses grow continually throughout the life of the animal (Smith, 1883), and Lord (1959) capitalized on this phenomena to work out the relationship between age and lens weight in cottontail rabbits. Since this first study there has been a great variety of research on this topic in other mammals (see Gosling, et al., 1981, for a recent review). A few birds have also been examined but the relationship is less useful in these animals (Dahlgren, et al., 1965). Evidently no turtle and only one reptile (common adder: Kheruvimov, et al., 1977) has been studied in this light.

Lord (1959) reported that weights of wet formalin preserved lenses showed a less predictable relationship to age than did dry lenses, and probably for this reason almost all subsequent research has been directed at dry weights of formalin preserved lenses.

The present study, a first step in a series of ongoing analyses, presents data on the relationship between fresh lens weight and age in 45 green sea turtles, Chelonia mydas (L.), all of known age.

## MATERIALS AND METHODS

Eye lenses were removed from 45 turtles ranging from 2.1 to 128 months of age. The total sample consisted of specimens from the following age classes: 128 mo - 1; 39 mo - 32; 15 mo - 5; and 3 mo - 7. All of these animals were hatched from eggs incubated at Cayman Turtle Farm (CTF), Grand Cayman Island (see Wood and Wood, in press, for a description of this facility). The oldest animal, CT-19 (CTF No. 536), came from eggs imported from Surinam. All other animals derived from eggs laid at the farm by Captive Wild Stock (CWS) and this sample included three age classes, depending on when they were hatched: C6 - 1978; C8 - 1980; and C9 - 1981. At CTF nesting extends from early August until December and, although the span in age between members of a single age class may be as much as 5 months, 80% of the eggs are laid in September and October.

Specimens were obtained by two means: salvaging animals that died, and salvaging parts of animals butchered in the normal activities of CTF. Lenses from the latter were usually weighed (and frozen for later analysis) within 4 hours of death. Time of death was not known for other animals, but tanks are checked regularly first thing in the morning (at 0700 hrs) and throughout the day until 1700 hrs; hence none of these animals is thought to have been dead for more than about 14 hours before the lenses were removed. In the few instances when eyes were necrotic, or lenses were cloudy, or blood was evident within the eye ball, the lens was discarded.

Lenses were prepared by first removing the contents of the eyeball. The orbit was cut around dorsally, posteriorly and ventrally separating exposed connective tissue until the eyeball could be pulled from the socket enough to make an incision in a vertical plane roughly bisecting the sclera into lateral

and medial halves. The vitreous humor with attached lens was gently scooped out and dropped into a petri dish. With lens uppermost, a circle was described with a # 11 scalpel, gently cutting the lens free from the ciliary body. The lens was then separated by pushing it away from the remainder, and cleaned by picking it up with a finger, rinsing it briefly in Lactated Ringers solution, and rolling it gently on a paper towel, picking off extraneous materials that stuck to the paper. The rinsing and rolling could be repeated. When clean, the lens was rolled for several seconds until superficially dry and weighed on weighing paper on a Sartorius electronic balance to 1/100 mg. After weighing, each lens was labeled and frozen covered in a solution of Lactated Ringers for later protein analysis.

## RESULTS

### Left-Right Asymmetry

Thirty-six pairs of lenses were weighed, and the greatest difference between the left and the right was usually less than 4% of the maximum weight, despite the side ( $\frac{\text{max}-\text{min}}{\text{max}} \times 100$ ). (In two cases, CT-1 and CT-20, the difference was 18% and 34% respectively; consequently the values for these two pairs were discarded). In 17 pairs the left lens was greater than the right, and there were no instances of both lenses weighing the same. The value of (left lens) - (right lens) varied from -0.26 to +0.37. Although the distribution of this value is rather flat, the mean of 0.01361 ( $\pm 0.14919$ ) ( $\bar{X} \pm 1$  St. Dev.) is not significantly different from 0 ( $t = 0.55$ , d.f. = 35,  $p > 0.05$ ). Hence, there is no evidence that the lens on one side of a pair is regularly larger.

### Fresh Lens Weight-Age Relationship

In 36 cases lens weight was calculated by averaging the left and right lens in a pair; in 7 other cases only one of a pair was weighed (usually because the other was inadvertently destroyed). Values for two pairs (CT-1 and CT-20) were discarded because of the unusually large differences between the left and right lenses in these pairs.

The relationship between fresh lens weight and age is shown in Figure 1. There is considerable dispute over the correct way to describe the statistical relationship of body measurements and age (Dapson, 1973; Gilbert, 1973), and we will take up this topic at another time.

Stepwise multiple regression was performed using six independent variables: Age, Age<sup>2</sup>, log Age, Body weight, Body weight<sup>2</sup>, log Body weight. Two sets of regressions were done using first log Lens weight and then Lens weight as dependent variables.

Predicting log Lens Weight, the model selected by the stepwise procedure is:

$$(1) \text{ Log Lens weight} = (-0.7645) - (0.0033) (\text{Age}) + (1.1215) (\text{Log Age});$$

$$R^2 = 0.9917.$$

Since there were several lens weight measurements for each of the first three age classes, lack of fit could be statistically examined (Draper and Smith, 1981). The conclusion that the model showed significant lack of fit ( $F_{1, 39} = 10.08, p < .01$ ) is further illustrated by a non-random distribution of the residuals around the fitted line.

Predicting Lens Weight, the model selected is:

$$(2) \text{ Lens Weight} = (-0.0419) + (0.2338) (\text{Age}) - (0.0009) (\text{Age})^2;$$

$$R^2 = 0.9818,$$

No lack of fit was found ( $F_{1, 39} = 1.42, p > .05$ ), and an examination of the residuals showed a random distribution around the fitted line.

#### DISCUSSION

Although the  $R^2$  (percent variation explained by the equation) of model 1 is high, close examination of the model indicates that the fit of the predicted values is inadequate. In addition, the equation is cumbersome, combining logarithmic and linear functions of Age.

In comparison, the  $R^2$  of the quadratic model (model 2) is slightly less (about 1%) than that of model 1; however, there is no evidence of lack of fit. Furthermore, the quadratic model is in a conventional format and hence is convenient to use; this model is preferred.

Model fitting is as much art as science, and an endless variety of permutations can be attempted. However, these preliminary results show that age and lens weight are very strongly related, and that body weight is less valuable as a predictor. Anyone familiar with growth in sea turtles will know that there is often tremendous variability in the body sizes of animals of the same age. In the present sample, animals 15(+2) months of age weighed from 0.9 to 5.6 kg, hence, the heaviest animal was 6 times greater than the lightest animal of the same age class. Yet, the eye lens weight of the lightest animal were close to the average weight for this cohort. A thorough statistical investigation of these data is currently under way.

The lens samples will also be analyzed further to investigate several other age related phenomena. Zigman and Yulo (1979) have shown that the proportion of aggregated proteins increases with age in the dogfish shark. This lab will be conducting similar analyses of these turtle lenses.

Bada and Masters (see review in Bada, et al., 1980) have reported that racemization ratios show age dependence not only in fossils but in certain recent organisms. An attempt will be made to analyze these turtle eye lenses for racemization ratios.

Paitigorsky (in press) has reported that turtle eye lenses not only have delta crystalline but also a new undescribed crystalline. His lab will be looking at the crystallines on these turtle lenses and possible age related changes in the proportion of the different proteins.

Full reports of these various protein studies will be presented when the analyses have been completed. Results to date show that fresh eye lens weight is an excellent indicator of age in sea turtles.

#### ACKNOWLEDGEMENTS

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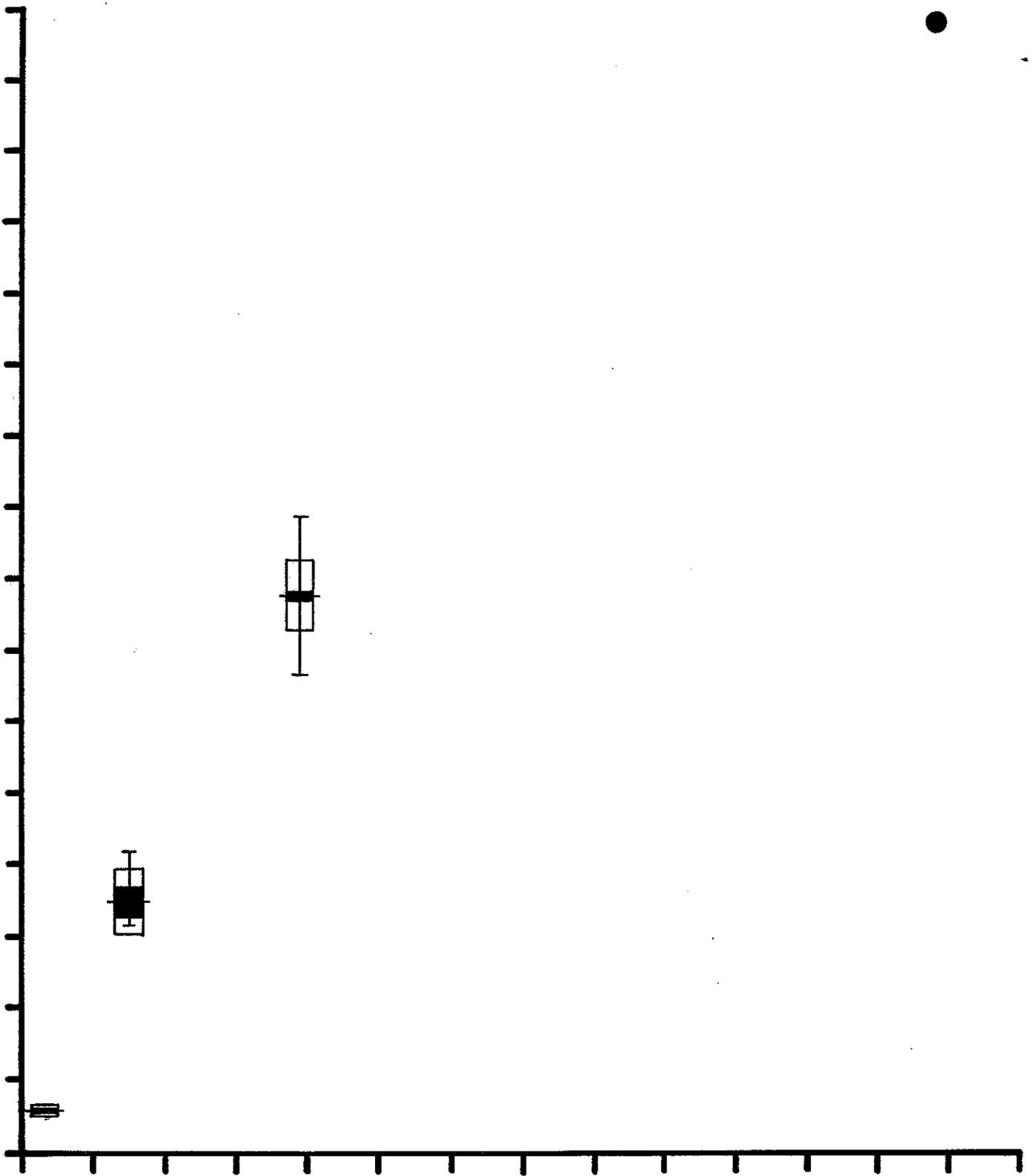
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## FIGURE LEGEND

Figure 1. The relationship between fresh lens weight and age in captive green sea turtles, Chelonia mydas (L.).

For each of the first three age classes, the mean (large horizontal line segment) is surrounded by  $\pm 1$  standard error (black area) and  $\pm 1$  standard deviation (open rectangles), and the vertical line shows the range of measurements.



## AGE RELATED CHANGES IN BLOOD CHEMISTRY

Discussion

Several age related changes are known in blood proteins and sterols, but these have not been applied to age determination problems. The findings are summarized here since these phenomena may be related to more conventional parameters used in age determination.

Phosphate compounds in red blood cells show age related changes in a number of bird and reptile species, including green and olive Ridley sea turtles, as well as two other aquatic turtle species. Three compounds show detectable changes: Concentrations of 2, 3 disphosphoglycerate (DPG) increase dramatically in late embryonic life, only to disappear at the age of about one year. Inositol pentaphosphate was first detectable in animals a few months old, and during the first year it essentially reaches adult levels. Adenosine triphosphate (ATP) levels drop at hatching and increase again during the first year to adult values (Bartlett, 1976; 1978). Although these changes are documented in a variety of organisms, the time periods during which they can be measured are too short to be provide useful means for age determination.

Blood serum cholesterol level, but not aortic calcification, has been reported to vary with body size (age) in the Suwannee terrapin (Pseudemys concinna suwanniensis) (Jackson, et al., 1970). The correlation, however, is unlikely to be an accurate estimator of age.

Age dependent changes occur in certain blood serum proteins. Gitlin (1975) has reviewed the "Normal biology of alpha-fetoprotein" (AFP) and described its prenatal synthesis in a variety of mammals and a few birds. As the half life of AFP is generally in the order of a few years, the analysis will have limited potential for age determination studies.

However, Patty (in prep.) is attempting to develop an age determination technique for great egrets based on AFP analysis. The potential of applying this technique to turtles is being investigated with Dr. Gitlin, and it may be possible in the future to do some pilot studies.

Since serum proteins can be stored deep frozen for some years without significant alterations (Frair, 1969; Wood and Wood, in press), it is possible to use frozen specimens.

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## AGE RELATED CHANGES IN COLLAGEN

Discussion

Studies in a variety of vertebrates have shown that there are marked changes in collagen correlated with age: it increases in quantity per unit area, insolubility (or stability) and tensile strength. Few reptiles have been studied, however.

Panigrahy, et al., (1978) worked with garden lizards (Calotes versicolor), reporting that solubility of hydroxyproline in collagen decreased with age; this was true with collagen from both bone and skin. Quantity of collagen per unit area of skin also increased with age. This indicates that collagen aging phenomena are common among vertebrates.

The value of the phenomena for age determination studies was shown by experiments done on breaking times of fibers of tail tendons in various rodents. Harrison, et al., (1978) and Bochantin and Mays (1981) showed that breaking times increase with an individual's age in various strains of laboratory and wild mice and rats. These values are also correlated with average maximum life span in different races of rats. Because Bochantin and Mays found that sample fibers could be stored for up to one month in distilled water at 4° C with no detectable effect on breaking time, the technique has great potential for field sampling.

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Smithsonian Aging Study: Loggerhead Sea Turtles (Caretta caretta) from Cumberland Island, Georgia.

### History

Since the early 1970's, Carol Ruckdeschel has regularly surveyed the beaches of Cumberland Island, Georgia, for sea turtle activity. Although the emphasis has been on a record of nesting activity, she maintained records of the frequency and the monthly distribution of stranded sea turtles (summarized in Ruckdeschel and Zug, 1982, Biol. Conservat., 22:5-9.). The regular stranding of large numbers of loggerheads and Carol's willingness to collect and prepare bone samples from them provided an opportunity to attempt an age determination of a single population.

The project began in 1978 with a literature review, and histological preparation of bony tissue to determine the best bone samples to retrieve from the stranded specimens. The presence of annuli (or growth increments) was found in the proximal limb elements, <sup>sporadically in cranial elements and distal limb elements,</sup> and not at all in the carapacial elements.<sup>2</sup> The presence of annuli in Caretta limb elements encouraged us with the feasibility of age determination.

The goals of the project are threefold: 1) to develop a method of measuring and analyzing bone annuli to yield a consistent and reasonable estimate of age in individual sea turtles; 2) with estimates of age, to develop an age-specific mortality chart for the stranded loggerheads of Cumberland Island; 3) to use the age estimates of Cumberland Island loggerheads to predict growth rates, age at sexual maturity, longevity, and other life history/demographic parameters for the loggerhead sea turtles of the southeastern United States.

Herein, we report our progress on the attainment of the first goal.

### Materials and Methods

Specimens.--The oceanic beaches of Cumberland Island were surveyed regularly from January through December in both 1979 and 1980 under FWS endangered and threatened species permit 2-2381. Every stranded sea turtle was individually numbered and measured (carapace length over the curve, carapace width over the curve, plastron length, tail length, cloacal position, head length, head width, *and* right front flipper length); the sex was determined when feasible; mutilations, injuries, parasitic load, and other features were noted; the date and locality of stranding was recorded; the stomach contents were removed (these data are being analyzed by Charles Potter and Carol Ruckdeschel); and the head and right forelimb were removed. The head and forelimb were macerated, cleaned, air dried, and stored as a dry skeletal preparation. The 1979 sample consisted of nearly 180 loggerheads and the 1980 sample of nearly 220 <sup>1</sup>loggerheads. Not all samples are complete, since many stranded turtles were missing their heads or flippers due to shark attack, apparent butchering by shrimpers, souvenir hunting by beach combers, or other causes. Nonetheless, the 1979 sample which this preliminary report is based upon will consist of well over 125 turtles.

Specimen preparation for microscopic examination.--The humerus was selected for sectioning and examination based on the initial study to determine which boney elements were most suitable. A series of measurements (see sample data sheet) are recorded from each humerus prior to sectioning. An approximately 5-7 mm thick transverse section of bone is cut with a <sup>b</sup>handsaw from the humerus just distal to the deltopectoral ridge. This section is stored in a 3% formalin

solution for at least 24 hours prior to histological preparation. The bone section is decalcified in a solution of equal parts of 8% formic acid and 8% hydrochloric acid for 4-8 days, depending largely on the thickness of the bone and its porosity. The decalcified bone is then rinsed in tap water and processed in a Fisher Histomatic Tissue Processor (model 166) as follows:

- 1) 95% ethanol for 24 hours, three changes of alcohol, and <sup>the</sup> last 12 hours under a vacuum;
  - 2) 100% ethanol for 30 hours, four changes of alcohol, and <sup>the</sup> last 18 hours under a vacuum;
  - 3) xylene for 12 hours, three changes, all under a vacuum;
  - 4) paraffin for 6 hours, two changes under a vacuum.
- The paraffin-impregnated bone is mounted in paraffin and transversely sectioned (6 micron-thick sections) on a standard microtome. All sections are stained in Harris hematoxylin and eosin. <sup>We should note that</sup> <sup>(3)</sup> during sectioning the bone should be periodically soaked with water to obtain the best results.

<sup>(4)</sup> Counting bone annuli.--The histological sections are examined at 60x magnification using a Wild micrometer. The length and width of each section is measured with a dial caliper to one-tenth of a millimeter. The periosteal annuli are counted and measured along two perpendicular axes, the long and short axes of the section, to an accuracy of one micron. Each annulus consists of a darkly- and lightly-staining band. Presumably the darker band represents an area of slow growth and the light band an area of faster growth. <sup>(5)</sup>

Age estimation.--Because the annuli of two axes are measured, it is possible to obtain two estimates of age for each specimen. Both are determined in the same manner. The widths of the annuli along an axis are summed and an average annulus width (AAW) is determined. Age is calculated by dividing the average annulus width by half of the bone axis <sup>distance</sup> (BA) taken from either the long or short axes of the section, minus the average diameter (1.51 mm) of the shaft

of a hatchling humerus (based on 16 hatchling specimens of Caretta caretta from Little Cumberland Island, Georgia). The formula is as follows: Age =  $(0.5 \text{ BA} - 1.51) / \text{AAW}$ .

### Results

Problem areas.--The standard decalcification and histological techniques did not yield consistent results for Caretta bone. Apparently the variable density as well as the compactness of annular structure prevents an even penetration and action of the decalcification agents, and similar problems for penetration of the histological fixatives and impregnation with paraffin. Several<sup>a</sup> decalcification agents (RDO, formic-hydrochloric acid, formic-sodium citrate) were tried. RDO is a fast-acting agent, so the variable density of the bone resulted in over-decalcification in some areas and under-decalcification in others. The formic-sodium citrate solution is slow and requires a period of several weeks to decalcify a section of bone and similarly produces uneven decalcification. The formic-hydrochloric acid solution produces relatively fast and even decalcification. We advise other researchers using our technique to keep the decalcifying agent constantly agitated and, if possible, to use X-ray for the determination of complete decalcification, until they have determined the average time of decalcification for their bone sections.

Even when the bone was totally decalcified, we initially encountered difficulties in sectioning. The bone was still hard and cut unevenly, tore, or came out of the paraffin block; obviously, the infiltration and penetration by the fixatives was uneven. A number of different protocols were attempted and the ones described in the Materials and Methods section yielded<sup>h</sup> the best

and most consistent results. In our experience, the use of an automatic processor is highly recommended. <sup>(6)</sup> Overall, it required nearly nine months of false starts and frustration <sup>^</sup> to develop a histological protocol that would consistently produce top-quality bone sections. This was undoubtedly a partial function of our inexperience, but also a result of the different nature of sea turtle bone. <sup>(7)</sup> We wish to acknowledge the assistance of the staff of the Armed Forces Institute of Pathology Bone Histology Section for their suggestions and encouragement, and particularly the help of Bud Cunningham and Janet Mancl.

Two difficulties <sup>^</sup> occur during the measurement of annuli; 1) the irregularity and lateral disappearance of annuli; 2) the presence of large areas of bone resorption and endosteal deposition. The size/age of the turtle influences the distinctiveness of the annuli. As a general rule, Caretta less than 700 mm carapace length (over the curve) have relatively evenly spaced and wide annuli, Caretta greater than 700 mm CL may show no annuli, a few vague annuli, or numerous narrow, densely packed annuli- the larger the turtle the less likely that annuli will be evident. To counteract the vagueness and irregularity of the annuli and, furthermore, to produce consistent results, we used the two axis measurement technique. This <sup>(8)</sup> permits two independent estimates of age and allows for an unevenness of annular development on different parts of the bone. Although annuli are sometimes lost laterally, we have found that our most consistent results are obtained from the long axis. This is due in part to porosity in the region of the deltopectoral ridge, and <sup>to</sup> the wavy nature of the annuli in the region of the muscle scar opposite to the deltopectoral ridge, in contrast to the relatively undisturbed compact bone found laterally on the bone.

Assumptions.-- The major assumption in age estimates based on bone annuli is the annual nature of each annulus. In other reptiles, each annulus has been shown to consist of an area of lightly staining (rapid growth) bone and an area of darkly staining (slow growth) bone that represent one year of deposition (Castanet, 1978, Acta Zool.(Stockh.), 59: 35-48; Castanet and Cheylan, 1979, Can. J. Zool., 57: 1649-1665; Hemelaar and Gelder, 1980, Netherlands J. Zool., 30: 129-135; Peabody, 1961, J. Morph., 108: 11-62). The species examined to date are temperate species and are exposed to alternating seasons of high and low temperatures which affect the growth rates of ectotherms. Caretta from the southeastern United States apparently utilize inshore waters as sub-adults, and also as juveniles and adults to some degree (Mendonça and Ehrhart, 1982, Copeia, 1982(1): 161-167), and thus are exposed to alternating periods of hot and cold. Hence, it is expected that bone deposition will show an annual pattern; however, this is not confirmed. Frazier (this report; Hohn and Frazier, 1979, Amer. Zool., 19<sup>no space</sup> (3): 953) present evidence from known-age specimens of sea turtles suggesting that each annulus represents one year. The evidence is, however, equivocal, such that the one annulus/one year hypothesis must not be accepted uncritically.

The ideal condition for age estimation would be to have a tree-ring analog where each periosteal annulus from birth to death is present. Bone is a very dynamic tissue, however, and all Caretta bone sections examined show an extensive area of resorption in the center. The earlier record of growth is lost and it is necessary to reconstruct/estimate the number of annuli lost. We have done this by obtaining an average width for all the annuli remaining and using this measure to estimate the number of

annuli lost. This method has two failings: 1) older/larger turtles show a compaction and narrowing of annuli which potentially will lead to a narrower average annulus width and an over-estimate of age; 2) ~~the~~ <sup>us</sup> annulus width undoubtedly increases as the turtle grows from hatchling to subadult, so the average width of subadult turtles will cause an underestimate of age. More accurate estimates of age will require the use of scaling factors in the age estimation formula. We have not done this for the present report; thus, our age estimate must be considered tentative and speculative.

In addition to the use of scaling factors to fine tune the age estimates, we plan to use growth data from natural populations (e.g., the Mosquito Lagoon population of Mendonça and Ehrhart, 1982) to cross-section our age estimate. We certainly do not believe that our aging protocol will ever obtain a one hundred percent accurate estimate, although we do believe that our final results will permit the development of a reasonably accurate demography and life table for the population of loggerheads from the Georgian coast in conjunction with the ecological data collected by the Georgia sea turtle group (e.g., Richardson et al.).

Tentative conclusions.-- The present data set consists of 67 observations and estimates. We will examine here a small subset of 21 individuals. In spite of the simplistic nature of our aging formula, the estimated ages show a strong correlation (linear  $r = 0.91$ ,  $p < .01$ ; power  $r = 0.90$ ,  $p < .01$ ) with humerus length (Fig. 1) and a slightly weaker correlation (linear  $r = 0.81$ ,  $p < .01$ ; power  $r = 0.82$ ,  $p < .01$ ) with carapace length (Fig. 2). The greater variation in the age-carapace regression suggested that the carapace measurements may have been subject to a greater measuring error. The bloating of stranded turtles often does distort shell measurements.

An examination of the humerus-carapace regression (Fig. 3) and the highly significant correlations (linear  $r = 0.96$ ,  $p < .01$ ; power  $r = 0.96$ ,  $p < .01$ ) does not confirm a greater measuring error. The alternate interpretation is simply that humerus length is a better predictor of age than carapace length. Possibly carapace length is a sexually dimorphic character and humerus length is not.

The nesting females of Cumberland Island have a minimum carapace length (over the curve) of 800 mm. If this size is used as an estimate of the average carapace length at which both males and females attain sexual maturity, the regression (Fig. 2) indicates that sexual maturity occurs between the ages of 20 to 60 years. The downward inflection of the power regression similarly supports such an interpretation. Even though we find these ages reasonable in respect to age at sexual maturity for the large emydine turtles of the southeastern United States and the slow growth rates of wild sea turtles, we do not encourage the reader to accept this for any more than a tentative estimate. We are, nevertheless, encouraged by the preliminary results and anticipate that this aging protocol will have practical value in understanding the demography of Caretta and in the development of management practices for the conservation of this species.

Project Personnel

- Carol Ruckdeschel: Cumberland Island; Research Collaborator, Department of Vertebrate Zoology.  
Stranding survey; collecting specimens and their skeletal preparation.
- Helen Wimer: Smithsonian Institution; Histotechnician, Department of Vertebrate Zoology.  
Histological preparation of bone samples.
- Addison Wynn: Smithsonian Institution; Research Technician, Department of Vertebrate Zoology.  
Gross sectioning of bone; data collection and analysis.
- George Zug: Smithsonian Institution; Curator, Department of Vertebrate Zoology.  
Principal investigator, ideally directing the research project and doing a little bit of everything.

Report prepared by George Zug and Addison Wynn

Species \_\_\_\_\_

Museum # \_\_\_\_\_

Sex \_\_\_\_\_

Carapace length

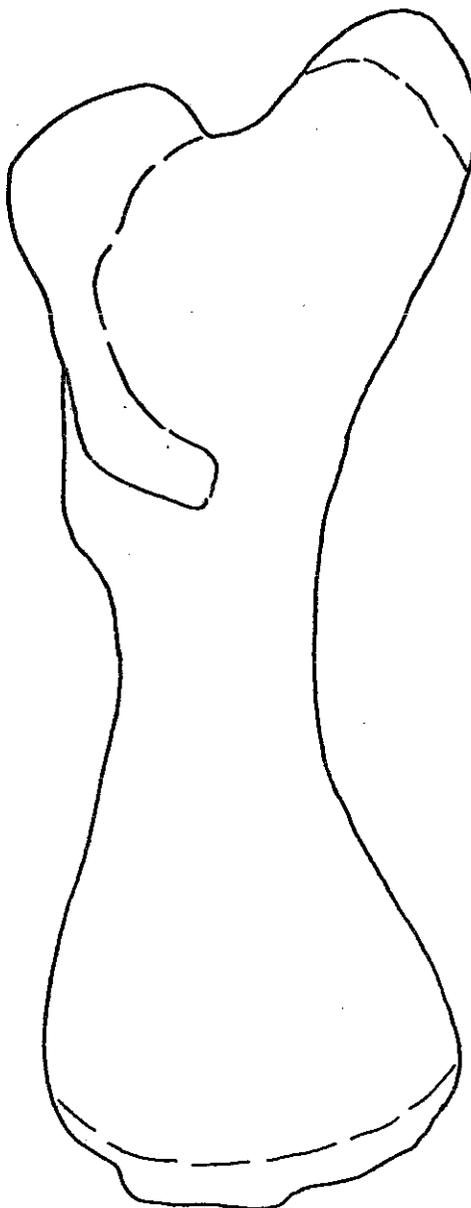
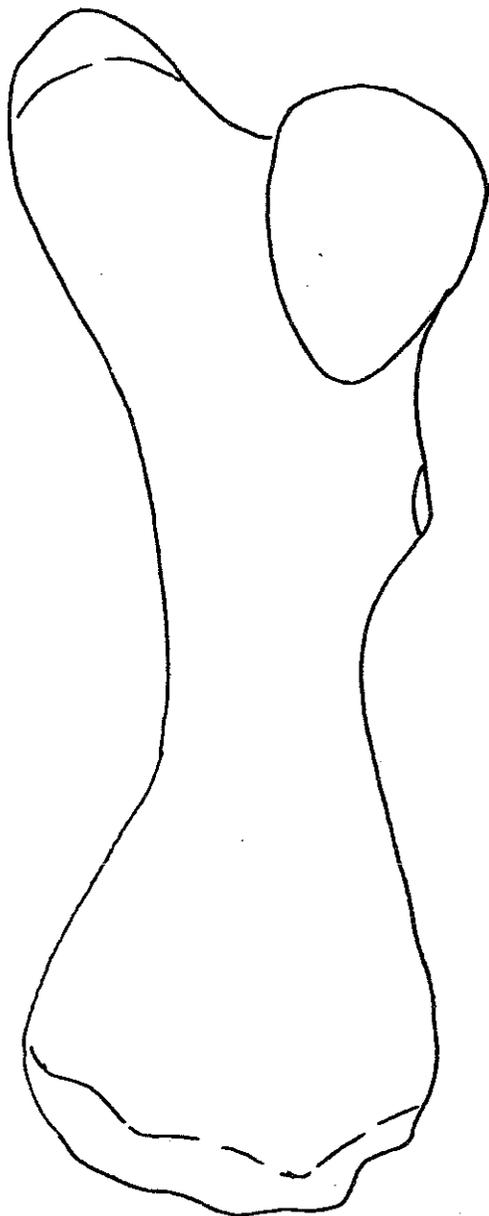
curve \_\_\_\_\_

straight \_\_\_\_\_

Carapace width

curve \_\_\_\_\_

straight \_\_\_\_\_



ML \_\_\_\_\_

LL \_\_\_\_\_

UPL \_\_\_\_\_

PL \_\_\_\_\_

PW \_\_\_\_\_

DPCL \_\_\_\_\_

DPPW \_\_\_\_\_

MW \_\_\_\_\_

DW \_\_\_\_\_

MaxHD \_\_\_\_\_

MinHD \_\_\_\_\_

T \_\_\_\_\_

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MEASUREMENTS OF SEA TURTLE HUMERUS:  
All Straight Line Distances in MM.

Measurements of sea turtle humerus: all straight line distances, in mm.

- ML, Maximum length: from proximal most tip of ulnar process to distal articular surface.
- LL, Longitudinal length: from proximal surface of head to distal articular surface (parallel to longit. axis of humerus).
- UPL, Ulnar process length: from proximal tip of ulnar process to juncture of head and process.
- PL, Proximal length: from proximal surface of head to distal edge of deltopectoral crest (paral. longit. axis).
- PW, Proximal width: from preaxial surface of head to postaxial surface of ulnar process (perpendicular to longit. axis).
- DPCL, Deltopectoral crest length: from preaxial to postaxial edge of crest (diagonal to longit. axis).
- DPPW, Width at deltopectoral process: from preaxial to postaxial surface at deltopectoral process (perpend. longit. axis).
- MW, Medial width: from preaxial to postaxial surface in middle of humerus at point of minimum width (perpend. longit. axis).
- DW, Distal width: from preaxial to postaxial surface at juncture of articular condyles with shaft (perpend. longit. axis).
- MaxHD, Maximum head diameter: across middle of head from dorsal to ventral surface.
- MinHD, Minimum head diameter: across middle of head from preaxial to postaxial surface.
- T, Thickness: minimum depth of humerus in middle of shaft, approximately in vicinity of MW (perpend longit axis & width axis).
- Wt, Weight: dry weight of humerus in grams.

Fig. 1- Estimated Caretta age plotted against humerus length. The best fitting line obtained by a linear function is shown with a dashed line, and the best fitting line obtained from a power function is shown with a dotted line.

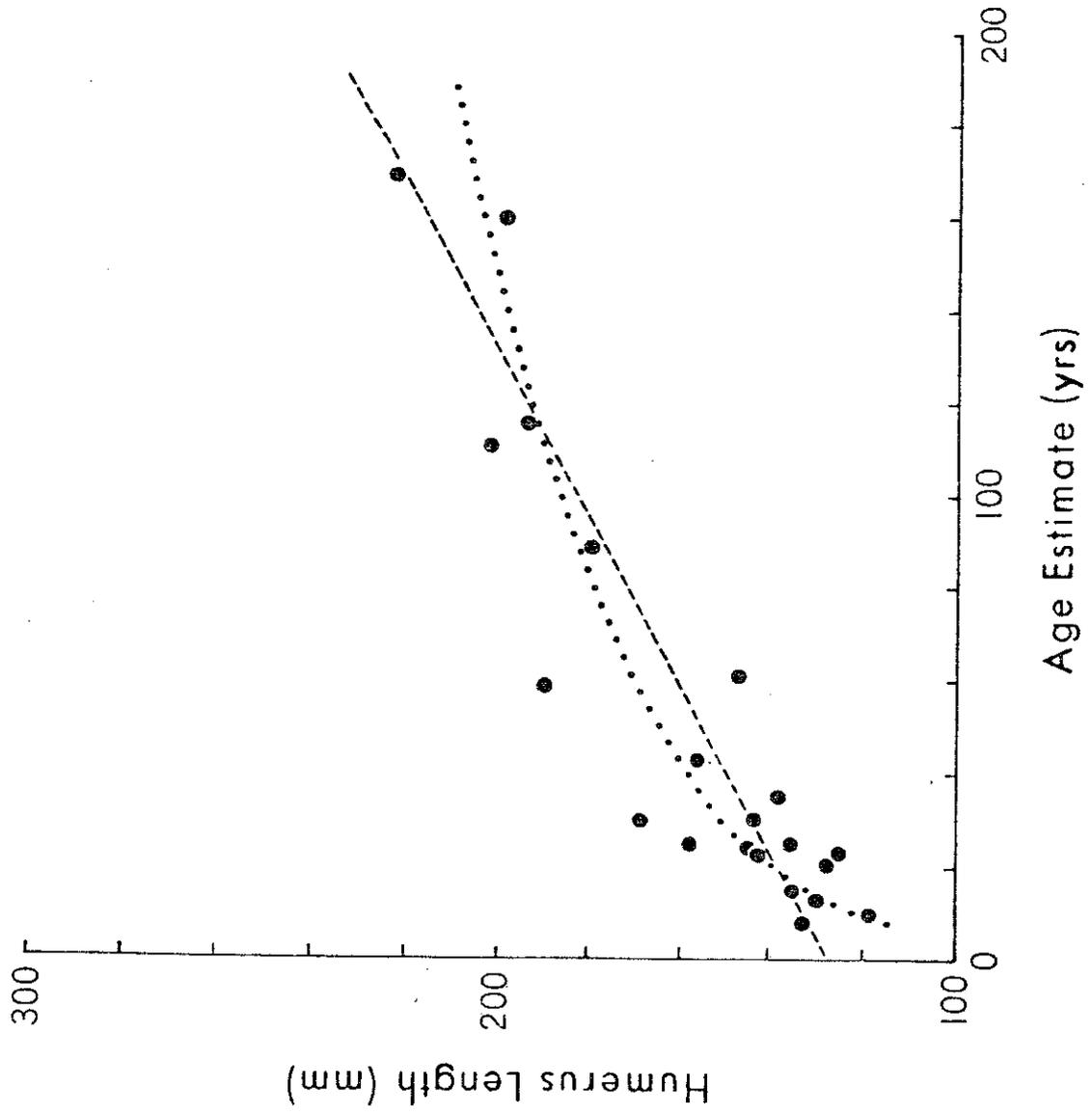


Fig. 2- Estimated Caretta age plotted against curved carapace length. The best fitting line obtained by a linear function is shown with a dashed line, and the best fitting line obtained from a power function is shown with a dotted line.

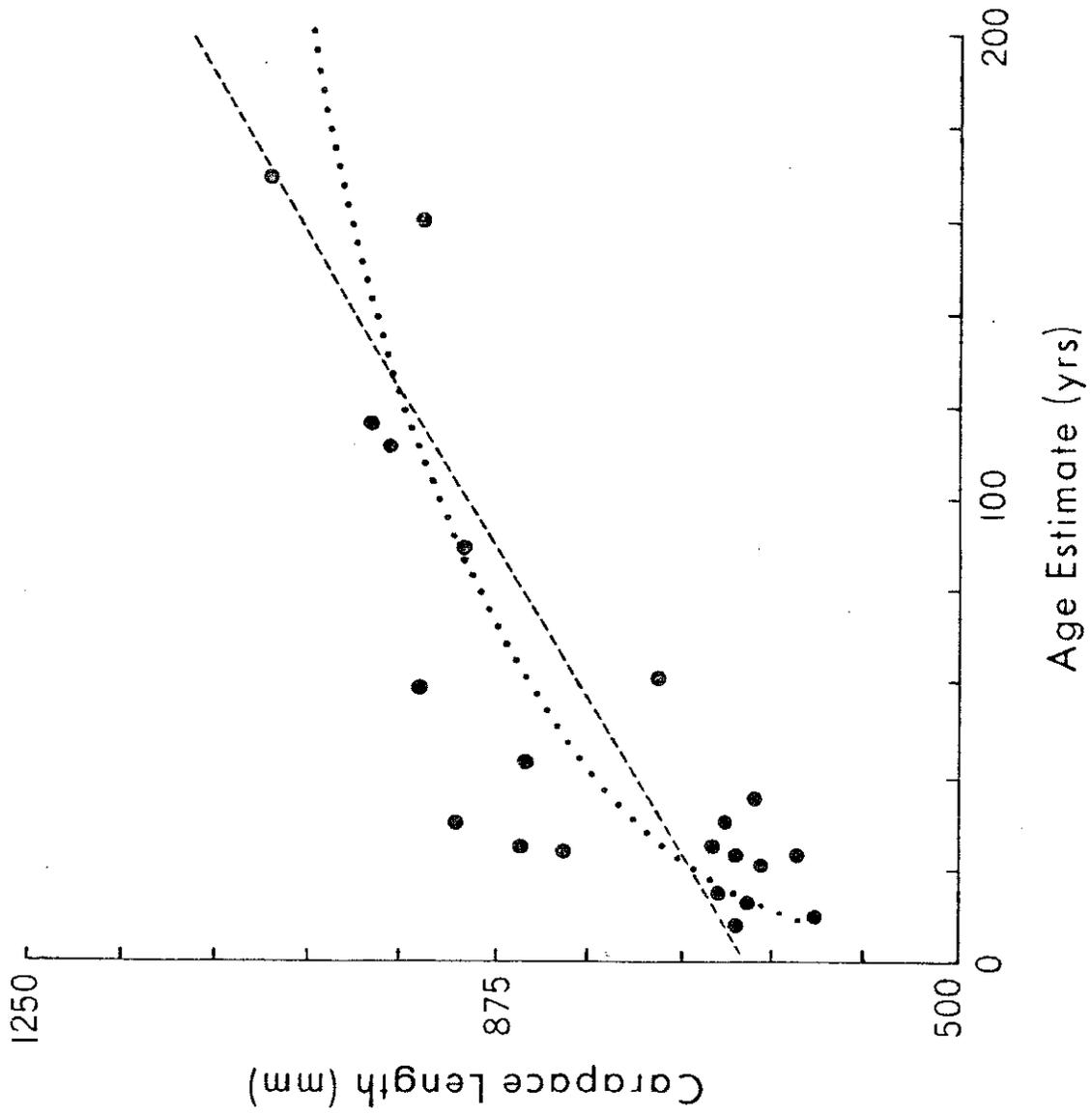
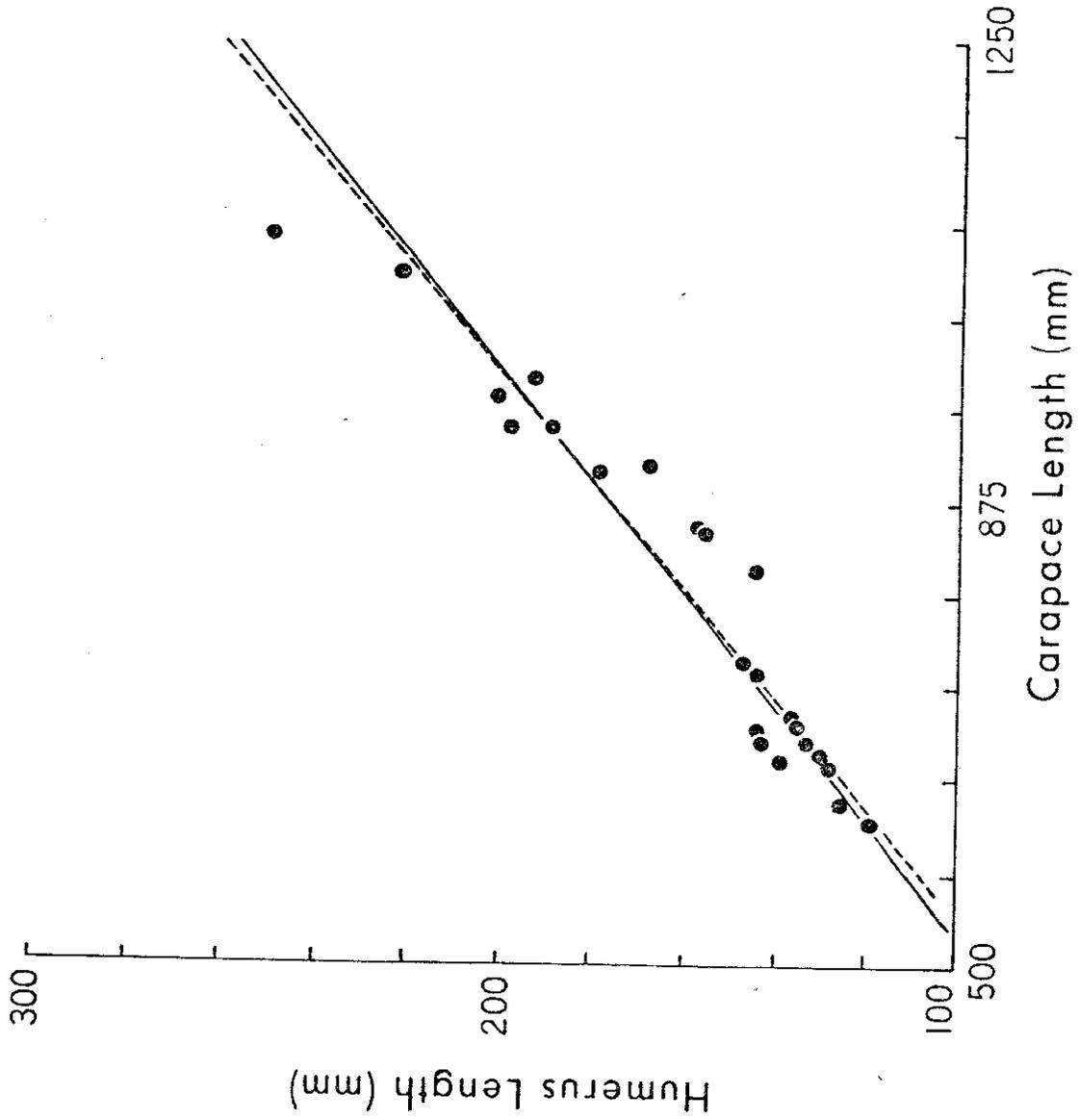


Fig. 3- Humerus length plotted against curved carapace length for Caretta. The best fitting line obtained by a linear function is shown with a dashed line, and the best fitting line obtained from a power function is shown with a solid line.



Footnotes by J. Frazier

- 1- This project originally had the collaboration of A. Hohn, an authority on dolphin aging, and J. Frazier.
- 2- Growth layers are occasionally visible in bones of the shell, but they are never either numerous or clear.
- 3- The bone exposed at the surface of the block being cut should be soaked.
- 4- The term "annuli" implies that the phenomenon is annual; "growth layer" does not carry these assumptions and is therefore preferred.
- 5- The terms "light band" and "dark band" may lead to confusion, for light and dark will change depending on transmitted or reflected light. "Opaque" and "Translucent" are preferable.
- 6- For the present purposes, the vacuum infiltration facility is the most important feature in the process.
- 7- Sea turtle bone, like other vertebrate bone, presents special problems for sectioning; however, the fact that it is sea turtle bone does not make it particularly more difficult than other vertebrate bone.
- 8- "Lateral disappearance" = (?) fusion of lines of arrest.
- 9- These estimates are different but not independent in the statistical sense.
- 10- This report infact questions this assumption.
- 11- "ML" in the data sheet.
- 12- It is common that carapace length varies greatly among members of a cohort.