A MORPHOLOGICAL AND EXPERIMENTAL STUDY OF THE GROWTH OF THE
SKELETON IN THE POSTERIOR EXTREMITY OF Gallus domesticus.

by

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Purpose of Studies.

It is established that the thickness and the arrangement of the architecture of bone in the adult is regulated to a large extent by the action of mechanical stress and strain. It has not been determined how much of a factor this is in the growing animal nor how early in embryonic development this regulating action develops. It has been maintained by one man recently (R. Thoma) that mechanical factors control the growth of bone from the start- that beginning with the first formation of bone, mechanical stress and strain control the process.

The present studies were started with a view of testing this portion of the problem, to find out if possible, whether the first formation of bone is dependent upon the action of mechanical forces, or whether it is regulated by some other factors. It was further designed to find out, in case bone were formed, whether it would have a normal architecture and a normal rate of growth during the early stages of development.

Review of Literature.

The view that the architecture of adult bones is regulated by stress and strain started with Meyer ('67), who, with the assistance of Culmann, a physicist, found that the head of the femur has an architecture made up of rods and bars so spaced and arranged as to furnish a maximum of strength with a minimum of material. The next important advance was made by W. Roux ('83),

who, studied the architecture of the knee joint of a man, who as the result of injury, had an ankylosis of this joint, with the femur and tibia fixed at right angles to one another. He found that there had developed an entirely new set of bony trabeculae, so arranged as to resist the stress and strain applied in the new direction, and again, such as to give a maximum of strength with a minimum of material.

In 1893 J. Wolf published a large monograph in which this thesis was worked out most elaborately, with careful studies of the normal, and studies on many cases of healed fractures and various diseased conditions. His general conclusions were that the architecture of the adult bone represents a response of bony tissue to the action of the mechanical factors of stress and strain- that with increase in these forces new bone is formed, while with decrease, bone is absorbed. As a result, bone is found where needed and only where needed, and in addition bone may change shape to meet new conditions.

Certain phases of the structure of the adult femur have recently been analyzed in a most searching manner by John C. Koch ('17), who had the advantages of a previous training as a mechanical engineer. He found to the smallest detail, an agreement between the arrangement of the trabeculae and the thickness of shaft, and the amount and direction of application of the load carried- an arrangement which would fulfil the demands of the most

exacting engineer, were he given the problem of providing, out of a minimum of material, a structure shaped like the human femur, with the maximum of strength for resisting the special stress and strain to which the femur is subjected.

An important contribution to the problem of bone growth has been made by R. Thoma, who analyzed many of the numerous studies of his predecessors and who attempted to formulate the laws of bone growth. Some of his laws are as follows:

Bone grows mainly by the addition of new layers and the resorption of old.

There is a limited amount of expansive growth in bone which has been laid down.

Growth of bone is regulated very largely, if not entirely, by the action of mechanical factors and in the following manner:

Growth in length of bone takes place when the stress and strain exceeds a certain minimum. Shortening of bone does not take place.

Growth in thickness of bone begins when the stress and strain exceeds a certain minimum, which Thoma estimates crudely as a force of 6.6 grams per square millimeter acting over twenty-four hours. With increased stress and strain there is increased rapidity of growth in thickness, until, after a certain maximum has passed, further increase in stress and strain causes absorp-

tion of bone. Absorption also takes place when the stress and strain is below the minimum.

Thoma ('07) goes much further than others in that he claims that, not only is bone growth in the adult and growing animals regulated by mechanical forces, but that the same applies to bone at its formation- that the very first ossification centers which appear, develop as the result of the action of stress and strain, and he calculates the possible mechanical factors, which, in the absence of any body weight and even in the absence of muscle contractions, are sufficient to cause the formation of bone. He does not however, carry his mechanical explanation back as far as the formation of the primitive cartilaginous skeleton. This, he admits, must, for the present at least, be considered as inherited. It is the inherited shape of the cartilage, he believes, which ultimately dominates the shape of the bone.

In claiming that all bone formation is the result of the action of mechanical factors, Thoma differs radically from Roux, who finds for bone, as for other tissues, that there are two main stages in the development of this tissue. First, there is the stage of primary 'self-differentiation'. Second, there is the stage of 'functional adaption'. In the second, growth is regulated by the extent to which it performs its special function, while in the first it takes place merely as a result of its inherited capacity for differentiation and growth- a process to which Noel Paton has given the name 'hereditary inertia'.

For the bone, however, Thoma gives no experimental or other conclusive proof for his claim that all bone growth from the beginning is regulated by mechanical factors, while Roux makes no attempt to determine when the first stage ends and the second begins.

An interesting observation was made by Weber ('51), which throws some doubt on Thoma's view. He found a new-born calf which had a complete absence of muscles in the posterior half of the body including the hind legs. This was evidently due to the absence of nerves, for the spinal cord and all nerves were missing posterior to the second cervical vertebra. In spite of this, which probably meant a reduction of all muscle pull from the normal forces acting on the bones of the hind limb, the bones were developed, although with ankylosed joints and weighing only about half as much as normally.

This finding of Weber's suggested the possibility of eliminating experimentally what will be shown to be the chief mechanical stress and strain exerted on the bones of the embryo, the result of muscle pull, and making an analysis of the extent of bone growth under such conditions.

For testing the problem, the chick was suggested because of the wealth of good material, ability to obtain the stages desired, rapidity of growth, and accessibility for operative procedures. The plan was to eliminate the muscle pull by interference with the nerve supply to the muscles of the legs, and to study the effect on the differentiation and growth of

the bones of the leg.

That the effect of muscle action is considerable in chick embryos has been shown by Preyer ('85), who found that chick embryos start to move during the fourth day of incubation and continue, intermittently, until hatching. E.R. and E. L. Clark ('14), analyzing the amount of movement, found that at six days a chick moves, in all, approximately six out of the twenty-four hours, while an eight day chick moves eight out of the twenty-four hours,- that the movements are violent, and comprise, at each stage, contractions of all the muscles in the body that are differentiated. Since the first bone to appear in the leg does not develop before the seventh day, and since movements of the leg start before this time, it is obvious that the muscle pull is a considerable mechanical factor, starting well before the beginning of the first ossification, continuing through this time, and on until hatching. And yet the mechanical effect of the contracting muscles was left out of account entirely by R. Thoma, who takes into consideration only the passive tension exerted by the skin, the muscles, and the other tissues, in estimating the amount of tension exerted on the cartilages.

The program laid out, then, was to attempt , if possible, to eliminate the movements in the leg of the chick embryo by suitable operations on the spinal cord at an early stage.

Duration of Movements in Chick Embryos

Adapted from E.L. & E.R. Clark
Jour. Exp. Zoology Vol. 17, p. 385

Constructed from record of
 operated chick

— see plate VI

Q. Satou

It was realized that the problem involved some method for an accurate comparison of the bones in the experimental chicks, with the normal, and also that a study of the shape, the architecture and the direction of stress in the adult were necessary, in order to properly appreciate the possible deviations in the experimental animals, as well as the possible appearance, in spite of operation, of hereditary factors.

While the program has not been carried through to the goal aimed for, owing principally to unforeseen difficulties with the operation, much of the preliminary work has been done, methods, for which in some cases modifications had to be devised, have been mastered and some interesting results have been obtained. The results bearing directly on the problem in hand are first recorded, to these are appended the results of observations and experiments of more remote interest.

Methods Employed.

The chief technical procedures employed were (1) the technic of operating, and (2) the technic of selective staining and clearing. Since these differ from the usual, well-known histological methods, they will be described in some detail.

1. The technic of the operations on chick embryos.

Since Gerlach ('87) first pointed out the availability of chick embryos for experimental procedures, various authors have described ways and means of operating on the embryos, closing the shell, and continuing incubation. Gerlach used a rather complicated saw- of the nature of a trephine- for opening the eggs. Following this he sealed on a metal collar and closed the egg with a glass cover slip. He used for this work of sealing a wax composed of, resin 3 parts, and bee's wax 2 parts. He used a 3% carbolic acid solution on his instruments and corrosive sublimate solution on his hands. As Rabaud ('08) found, the simple wax devised by Gerlach is most satisfactory. The clumsy cells used by him- the embryoskop- are now however replaced with slips of mica. In the experimental work on chick embryos at the University of Missouri, the technic used is that of Professor Clark, with modifications necessary for the immediate problems. This has been described recently by Chapman ('18). The materials needed are a suitable 'warm Box', a binocular microscope, an arc lamp, sterile Ringer's solution, thin slips of mica, small forceps and

needles and an iridectomy scissors. The entire procedure may be carried out at room temperature, especially in warm weather. The best results were obtained by using the warm box. The warm box is constructed with an opening for a binocular microscope, with openings for the hands, and with one side of glass permitting suitable illumination of the embryo with an arc-light.

The work divides itself into two phases, (1) the opening and closing of the shell, and (2) the actual work upon the chick. The latter will be described later in the paper when discussing the operations. The eggs were held in front of a light to determine their fertility and to see if the embryos were adherent; ^{if they were,} the eggs were rotated until the yolk turned freely. In opening the eggs and in handling the chicks, asepsis was carried out as far as possible. The coarser instruments were passed through a flame, the more delicate ones were dipped in alcohol. The egg itself was swabbed with alcohol over the area to be opened. A small hole was scraped through the shell with the back of the point of a scalpel. A drop of Ringer's solution was added to loosen the shell membrane and the shell over the embryo was removed by biting out small bits with a forceps.

The use of Ringer's solution was found to prevent the blastoderm from sticking, with danger of tearing. It was impossible to begin operations on the chick immediately upon opening the egg, for the embryo was found to sink below the surface.

This was obviated by the addition of Ringer's solution, a few drops at a time. In from five to eight minutes the chick ceased to sink. An opening was then made in the vitelline membrane and the amnion was parted. This satisfactorily exposed the embryos for operation. After the operation Ringer's solution was added until it was slightly heaped up so as to exclude air bubbles. A mica disc was applied and sealed on with Gerlach's wax. The mica was always flamed to prevent infecting the chick. No air was left over the chick. With even moderate asepsis there is little danger of infecting the embryo. The egg was returned to the incubator and, for the best results, rotated every fifteen minutes during the first three hours to prevent the disturbed parts from adhering to shell and thus hinder free movement which was necessary for repeated observations through the opening.

2. The Staining Technic.

Anticipating the importance of being able to make accurate comparisons between the bone and cartilage in the operated animals and the normal, much time was spent in acquiring the technic of selective staining and clearing in toto, since it was thought that a more precise comparison would be possible if distortions due to embedding and sectioning were avoided. In developing this technic, certain modifications from the descriptions as given in the literature were found to be necessary and it is therefore given in considerable detail.

The "Schultze" method of clearing tissues with potash solutions for the study of the calcified portions of a skeleton has been used for some time. This procedure is rapid and fairly satisfactory as far as the ossified parts are concerned, but relations between bone and cartilage are not evident. In this study several stages of the chicks were cleared by the potash method for comparison with the method of double staining. The potash method of Nelson, quoted by Guyer ('14), was used. This consists in placing the freshly killed specimens in a one per cent solution of potassium hydroxide over night and then transferring them to glycerine. This method is in no way comparable to the methods described below. I have seen the original specimens prepared for Professor Lillie's embryology by Roy L. Moodie and even these fall far short of specimens desirable for topographical study or for demonstration.

The methods of rendering tissues transparent with substances of high refractive indices have attracted attention for several years. In principle a substance is sought which will have the same refractive index as the dehydrated specimen. Concomitantly, methods for selective staining of bone and cartilage have been developed. The question of priority does not concern us here and it is therefore sufficient to refer to the work of Spalteholz ('14) and Lundvall ('04 & '05), who have made the most important contributions. Spalteholz appends a very complete

bibliography to his latest pamphlet ('14). Since the appearance of the pamphlet (the '14 edition is the second), various technics for staining bone have appeared, yet they all seem to be but repetitions of Spalteholz's work. Spalteholz in turn took advantage of the old experiments with madder feeding. (Alizarin is a component of the madder root.) Blumenbach (1815) mentions the following: "Dass die Knochen mancher Thiere nach dem Genuss der Färberröthe roth werden, hat schon Laevin. Lemnius in der Mitte des XVI Jahr. angemerkt. d. defs. miracula occulta naturae p. 390 der Antwerp. Ausg. von 1581. 8."

The English translation of 1827 (Lawrence and Coulson) says:

"The red tint, which bones of animals receive in consequence of madder being mixed with food, is observed by Ant. Misaud in his Centuriae Memorabilium seu Arcanorum omnis generis, p. 161. Cologne, 1572, 12mo". Belchier (1736) is the earliest author mentioned in recent literature (Brooks '17). Brooks quotes Beckman as citing evidence that this fact of the coloring power of madder root was probably known to the ancients. Both Lundall and Spalteholz consider the selective staining of bone, in addition Lundvall describes a method for the staining of cartilage, as well as one for the differential staining of cartilage and bone. The method here adopted is a combination and slight modification of the methods of the two authors. The percentage of the alcohols were

altered so as to lessen the probability of shrinkage. In general, the staining methods of Lundvall and the clearing methods of Spalteholz were followed. For the early stages in which limb structures were in the 'prechondral stage', the toluidin blue method was found superior. In the later stages of development the presence of the calcium containing feathers complicated matters since they stain heavily with the alizarin which was used for staining bone. In some of these specimens decolorization was effected with a one per cent solution of hydrogen peroxide and in some the skin was removed.

For the staining of the cartilaginous skeleton the following was used:

1. Fixation in 10% Formol.
2. 50% Alcohol, 6-24 hours.
3. 70% Alcohol, 6-12 hours.
4. Toluidin blue solution, about 6 hours.

(0.25 grams toluidin blue in 100 cc. of 70% Alcohol.

Let stand over night, add 1.0 cc. HCl and filter.)

5. Decolorize in a $\frac{1}{4}$ % solution of hydrochloric acid in 70% alcohol.
6. Dehydrate thoroughly and clear as below.

(With the exception of the clearing, this is the method as described by Lundvall.)

The importance of this stain lies in the fact that the cartilage retains the toluidin blue longer than the other tissues, when acid alcohol is used, so that, after clearing, all the cartilage present is stained a deep bluish green. This enables the cartilage to

be clearly seen while the other tissues remain unstained and transparent.

For differential staining of bone and cartilage.

1. After thorough bleeding, wash the specimen in normal saline and fix in 60% alcohol. (Fixation in formaldehyde is recommended, this to be followed by bleaching in peroxide of hydrogen. Unless the peroxide is thoroughly washed out, poor staining results.)

2. For staining the bone in large objects, use solution A given below. Use solution B for small ones. Optimum time 8 hours.

Stock solution. Saturate 70% alcohol with sodium alizarin monosulphonate and filter.

Solution A. -1 part stock solution, 19 parts 70% alcohol

Solution B. -1 part stock solution, 9 parts 70% alcohol

3. Wash in 70 % alcohol.

4. Stain for cartilage in methyl green from 6 to 12 hours.

Methyl green	1 gram.
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70% Alcohol	200 cc.
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Glacial acetic acid	2 drops.
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5. Decolorize in 70% alcohol by changing until no color is in the alcohol after standing over night.

6. Dehydrate by running through the usual alcohols and finish with two changes of good absolute.

7. Clear by placing the object in benzol until it sinks,

then transfer to the artificial oil of wintergreen.

(methyl salicylate).

Notes: All reagents should be neutral- this is very important since the presence of acid, except for the trace of acetic acid in the methyl green staining solution, decolorizes too much, while the presence of alkali results in a general staining of the tissues with alizarin, which it is impossible to remove. If the formaldehyde is not thoroughly removed, a general purple red staining of all tissues results, which likewise cannot be removed.

With proper precautions, the results of this method are specimens with the cartilages stained bluish green, bones red, while the other tissues are unstained (or faintly purple), and transparent, so that the bone and cartilage can be clearly seen. The staining of the bone is not so dense as to prevent seeing the details of the architecture.

The method represents a combination of methods recommended by both Lundvall and Spalteholz- with deviations- particularly in the use of 70% instead of 95% alcohol for the alizarin staining. For decolorizing, Lundvall alternates between 70% and 95% alcohol, a method which was found to be unnecessary.

The Chemicals Employed in Staining.

Owing to the fact that it is impossible always to obtain chemicals from the same manufacturer, the following formulae and trade names of the various substances were compiled and are appended for reference.

The Cartilage Stains.

Brodesen ('15) says that the Grubler people give the following formula for their "Toluidin blau":

Draeper and Feilman in Vol. V of Allen's Commercial Organic Analysis p. 280 give the following formula for methyl green:

Bone Stains.

The same authors, p. 220, give the following names as applying to Sodium Alizarin Mono-sulphonate:

Alizarin Red S

Alizarin Carmine

Alizarin WS

In addition to these, the more chemical names given below are frequently met with:

Alizarinsulfosaur Natron

Alizarinsulfosaures Natrium

Sodium Alizarin Sulphonate

The formula for the above, according to Fay, ('11) is:

Notes: The sublimed alizarin is obtained from the alizarin paste used in the dyeing industry. It is unsuitable for use because of its low solubility.

Alcoholic solutions of the madder root likewise do not give satisfaction in staining results.

The Skeleton of the Posterior Extremity of the Adult Fowl.

Soon after commencing the experiments, it was realized that an accurate knowledge of the adult skeleton of the posterior extremity, particularly of the femur, was desirable, in order to interpret the results of the experiments. It was considered important to study the shape of the bones, the architecture, and the directions along which the mechanical forces of stress and strain are exerted. This is especially important in view of the possibility of the appearance in the embryonic bones of a shape and structure which result from the action of heredity, rather than from the action of mechanical factors, and which, if such proved to be the case, would appear, even in the absence of the pull of the muscles.

Studies were therefore made of a number of adult skeletons, with special emphasis on the femur. The architecture was studied by sawing through the femur longitudinally, both in the frontal and sagittal planes. It was possible to follow the curve of the femur by using a jeweler's saw, the very fine blade of which, makes a clean-cut surface, with a minimum loss of material. In order to find the position and direction of the bones in the normal standing position, an x-ray was taken of an adult hen in a natural, standing position.

A brief general description will first be given of the entire skeleton, taken mainly from the literature, and this

followed by special observations on the femur.

The most complete account available of the structure of the adult skeleton of the hind limb was that found in Vaughn's handbook ('76). A short description is given by Bradley ('15) and a summary by McFadyean ('08). Bronn's Tierreich ('91) and the various articles of Shufeldt are useful in familiarising oneself with the terminology of avian osteology.

The skeleton of the bird is peculiar in that there are no epiphyses. Furthermore the pubes do not unite in a symphysis, therefore strictly speaking the term "pelvic girdle" should not be used. For this reason the elements comprising the pelvis will be here termed the "pelvic arch". The problem of the ossification of the tendons and likewise the growth of the spurs in the male are not considered here. In the following description, Vaughn has been followed rather closely.

The Pelvic Arch. The pelvis by extensive fusion with the vertebral column presents a strong structure counteracting the weakening effect of the absence of a symphysis. The ilium extends over the last thoracic vertebra and terminates posteriorly on a line with the fifth coccygeal vertebra. All except the posterior portion is firmly united to the vertebral column. Anteriorly, two foramina are formed by the fusion of the two iliac bones with the spinous processes and by the extension and fusion of the transverse processes. The inferior edge of the ilium is

free along its anterior part, then it forms a part of the acetabulum next the superior border of the sciatic foramen and posteriorly it is united with the ischium. The superior face of the ilium slanting forward and downward presents an extensive attachment for the gluteal muscles. In the acetabulum the anti-trochanteric surface articulates with the femur.

The ischium is narrow and thick at its anterior end where it lies in the posterior part of the acetabulum. It forms the inferior border of the sciatic foramen and back of that unites with the ilium. The ischium is triangular in shape with the base posteriorly.

The pubis is the smallest bone of the group. It forms the lower part of the acetabulum and a little posteriorly it unites with the ischium to form the foramen ovale and ends a short distance from this in a blunt tip.

The Femur. The shaft of the femur is cylindrical in shape with a decided forward curve and a lesser lateral one. The proximal end, while resembling the mammalian structure in general, presents some marked differences. The neck which is short and thick extends at right angles to the shaft. The head is hemispherical and presents the usual roughening at the point of attachment of the ligamentum teres. The head is much too small to fit the acetabulum and as a result the medial side of the trochanter has an articular surface which is continuous with that of the head.

This space between the head and the trochanter forms the main point of support for the weight of the body. The trochanter extends upward from the neck and is pointed medianward.

The distal end of the femur has two prominent condyles separated by a median fossa, which is convex longitudinally and concave laterally. The lateral condyle is the larger of the two and it is divided posteriorly by a groove into a large medial portion and a smaller lateral one. The medial portion is articulated with the tibia, while the groove and the lateral division unite with the fibula. The architecture will be taken up later.

The Patella. The patella is a small irregularly shaped bone imbedded in the strong broad ligaments of the femur and lying in the intercondylar fossa. Convexities corresponding to the internal faces of the corresponding condyles on the femur are found.

The Tibia. The tibia, the largest bone in the body, in reality is the tibia-tarsus as appears from a study of its development. The distal end is fused with a row of tarsal bones. The proximal end has two articular surfaces which are separated by a ridge known as the intercondylar convexity. Anterior to the articular surfaces is a crest which rises above these surfaces and extends transversely. The anterior face of the crest is marked by a thin crooked ridge. The external side presents two roughenings in the longitudinal direction indicating the articulation with the fibula.

The Fibula. The fibula is a small bone on the lateral side of the tibia and attached to it by fibrous tissue. It has a head which is flattened laterally, points backwards, and has a convex anterior surface. The fibula becomes smaller from above downwards and terminates in a spicule of bone about four-fifths the length of the tibia. This spicule is continued downwards as a tendon and is inserted into the lateral condyle of the tibia. Well-marked roughenings indicate the attachment of the fibula to the tibia. This attachment occurs for about one-third the length of the bone- generally an attachment at the head- a hiatus- and then a continuation of the attachment.

The Metatarsus. The tarso-metatarsus, sometimes called the metarse, and perhaps most frequently the metatarsus, is, as a study of its development shows, a composite bone. The upper extremity has two articular surfaces, the entocondylar and the ectocondylar. These are divided by the intercondylar space. From the inferior part of this space a small protuberance arises which is said to act as a brace while the bird is roosting. In the male about two-thirds of the way down the medial side of the bone a short conical projection, curving slightly upwards serves for the support of a spur. The rudimentary first metatarsus bone is connected by a ligament to the inner and posterior part of the distal portion of the common bone.

The Phalanges. The metatarsus articulates with four digits, three anterior and one posterior. The posterior or first digit has two phalanges, the second three, the third four, and the fourth five. The third toe is the largest and the first is the smallest.

anterior →

parallel with standing base

A study of the position of the bones in the x-ray picture of the hen in the normal standing posture, shows that the various parts of the leg make the following angles with the vertical: the femur points forward making an angle of 65° with the vertical; the tibia-tarsus points backward at an angle of 25° with the vertical; the metatarsus points forward again, at an angle with the vertical of 20° . It is interesting that the angle between the femur and the tibia is exactly 90° .

The most striking feature of the posture is the deviation of the femur from the vertical. From this one would expect an internal architecture showing the result of the stress and strain on the femur in this position. If a frontal section is made, the trabeculae in the two ends of the bone are seen to form a coarse meshwork, made up of two chief sets- the one set perpendicular to the joint surface, the other parallel to the joint surface and perpendicular to the first set. This arrangement agrees, in general, with the arrangement of trabeculae found in the bones of the human skeleton where it is found that there are two sets of trabeculae, one set perpendicular to the surface and the second at right angles to the first.

Somewhat more interesting, particularly in connection with the wide deviation from the vertical which the femur makes in the standing position, is the arrangement as seen in a sagittal section. This reveals, in the distal end of the femur, a strong series of trabeculae running from the articular portion of the

Angles Formed by leg Bones of
the Adult Fowl
constructed from Plate II

W. S. S. S.

Antero-posterior section of Femur (rt)
of Chicken - ♂ - age about 3 yrs.

Lines indicate direction of
trabeculae - more
apparent in fresh.

Fig 1 (X 1.2)

Femur in same position as
in Standing.

Side-To-side section
of femur (lt) from same
fowl.

Direction of trabeculae
indicated.

Fig 2 (X 1.2)

W. Watson

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condyle to the anterior surface of the shaft. If the direction of these is considered with reference to the position of the femur in the normal standing posture, these trabeculae are seen to be perpendicular to the horizontal plane. In the proximal end a similar, although lighter group of trabeculae, runs from the articular surface to the posterior side of the shaft- again in a direction nearly perpendicular to the horizontal, when the femur is in its normal position in the body. These two sets are clearly arranged asymmetrically as a result of the oblique direction of the femur, and represent the response of the bone to weight exerted at an angle, rather than directly along the shaft. The specimens show the trabeculae much better than the photographic reproductions. The points made however are perfectly apparent from the photographs.

Experiments on Chick Embryos.

The results of experiments which were designed to eliminate movements in chick embryos, in order to study the early growth of bone in the absence of the mechanical effects of muscular activity, together with the results of experiments on related work that developed with the problem, will now be described. On the basis of studies which had been made on frog embryos by Harrison ('04) and his pupil Hooker ('11), in which immobility was produced by extirpation of parts or all of the spinal cord, it was thought that similar results could be readily obtained in chick embryos. This idea received encouragement from the fact that the movements of chick embryos, to which reference has already been made, are so widespread, and apparently so well correlated- such movements, for instance, as alternate swaying of the body and tail from one side to the other, with inward and outward movements of wing and leg buds with periods of activity alternating with periods of rest, - that it was inferred that they were regulated by the central nervous system.

The first experiment planned was the simple one of making a single transverse cut through the spinal cord, expecting thus to immobilize the legs. For this operation it was found after repeated trials that the seventy-two hour stage seemed to be best adapted. The cord was severed by a transverse cut through the epithelium, spinal cord, and notochord of the chick.

In the early experiments owing partly to the fact that the technic of working under the binocular was being developed, the mortality was very high. Many of the chicks did not survive the operative procedure and many others died in a few hours because of bleeding from punctures of the aorta, puncturing of the yolk membrane, as well as from undetermined causes. Eventually a fair percentage of the operations were successful, although the mortality percentage always remained high.

When these chicks were examined for movement, by watching them through the window in the egg, they were seen to present, much to my surprise, the usual type of movements. Periods of rest alternated with periods of activity, in normal tempo, and the movements involved all parts of the embryo, with no break between the anterior and posterior portions. Upon allowing several of them to hatch, they were found to be normal with no impairment whatever in the movement of the legs. Superficial examination failed to show a scar upon the back, or a deficiency in the vertebrae. It is therefore clear, that after cutting through the cord at three days, continuity is reestablished, and function is not interfered with. At the time that this was done Hooker's work ('15) upon the regeneration of the cord in the frog embryo had not been published, and, as previously stated, considering the extent of the movements of the chick it was thought that this would have been sufficient.

The results of this series show in part, for the chick what Hooker has demonstrated for the amphibian, viz. "Regenerative processes in the spinal cord severed in the cervical region in frog embryos at the stage following the closure of the neural folds will reestablish its anatomical and physiological continuity under favorable circumstances." The work on the frog included a careful microscopic study of the regenerative process. As the chicks were not examined in detail nothing can be said of the microscopic picture accompanying the regeneration.

Next it was planned to eliminate this regeneration by removing a section of the cord for a distance of several segments. In this series a portion of the cord varying from three to six segments in length was operated upon. Here again the technic of removal had to be perfected before results could be looked for. In all of this work the instruments had to be sharpened under the binocular microscope. The needles used were sharpened in several ways, some were prepared as very thin spatulae, others had a cutting edge, and still others were sharpened to a very fine point. The forceps proved to be the most useful instrument. A very delicate type was selected and then carefully sharpened. The points when finished almost equalled those of the needles in fineness and with this they met at the tips and were capable of biting. For removing several segments, the forceps were placed under the left side of the neural tube. From the right side a needle was

Plate V

Chick at seven days incubation following a transverse cut of the cord at three days incubation.

At 72 hours incubation a transverse cut was made completely severing the neural tube and exposing the notochord at the level of the omphalomesenteric vessels. From the time of hatching, the chick showed no abnormality in its movements. In the picture the blurr of the left foot was produced by movement. The chick was killed at 9 days. The only gross lesion was a 1 mm. defect in the vertebral column at the level of the 3rd thoracic vertebra.

introduced below the neural tube so as to pass between the slightly separated points of the forceps on the opposite side. In doing this, the aorta had to be avoided. In this way the cord was separated for the distance desired and then lifted out with the forceps. It took some time to develop the coordination that this required, but finally as shown by the higher powers of the binocular several segments of the cord between the two limb buds could be completely removed. These chicks, like those in the first series, moved in a normal manner. Upon allowing two of these to hatch, one specimen was obtained that had no demonstrable physiological connection between the anterior and posterior part of its body. In the other, slight connection seemed to exist, for violent movements of the wings were accompanied by movements in the legs. Stimulation of the legs resulted in a movement above the cut, as well as chirping. In the other chick no amount of stimulation of the hind limbs would give responses anterior to the cut. Stimulation of a toe, however, would give slow walking reflexes. At times, clonic contractions of the limbs were seen. Spontaneous movements in the limbs were observed shortly after hatching. The back of this chick showed a slightly elevated area of tissue which was apparently different from the epithelium. Upon the death of the chick which occurred on the sixth day, the skin was removed and this structure was found to be the spinal cord. It came to the surface through an opening in the vertebral column. At this point the cord was flattened but was continuous with a normally appearing

cord above and below. No microscopic examination of the cord at this point was made. As regards the isolation of the nervous control of this posterior part of the body, there are two possibilities: either the structures in the cord were not physiologically continuous or the exposure of the cord at the surface inhibited impulses. The latter view seems the most probable for movements were present shortly after hatching. Later experiments seem to point to this view as will be indicated in the next series of experiments.

Since this last series suggested a rather extensive regeneration, it was decided to increase the length of the cord removed. This gave the same results as before, possibly due, in part, to an incomplete removal of the cord, or possibly to the multiplication of the rather undifferentiated elements in the caudal portion. According to Ramon Y Cajal quoted by Lillie ('08) the formation of fibers in the cord does not begin until nearly the end of the third day. This no doubt accounts for the ease with which the structure was found to regenerate.

It was therefore decided to attempt a still more radical operation on the cord. The operation just described was modified so as to include the attempted removal of the entire cord posterior to the wing bud and clipping off of the tail with a pair of iridectomy scissors so as to remove that portion of the nervous system which could not be removed in the usual way.

In this operation the technic for the removal of a few segments was followed. It was found also that by inserting the closed forceps under the neural tube on the right side, and then carefully allowing the forceps to open, fairly satisfactory results were obtained. This procedure apparently mutilated the myotomes more than the other but I believe it resulted in a more complete removal of the nervous tissue.

The mortality was highest in this series, owing to the extent of the operation, nevertheless, several successful operations were obtained. While it was difficult to be certain, at the time of the operation, whether all the nervous tissue had been removed, still, it could be definitely seen that the dorsal portion was removed for long stretches, and that the continuity was broken in many places.

It was, therefore, a great surprise to find that, in each chick which survived the operation, movements started and continued as in normal chicks. One of this series was especially carefully studied, and a description follows.

The chick was operated upon at eighty-two hours- a stage slightly older than that at which nerves start to grow out into the leg. The operation was thought to be particularly complete- beginning at the region of the wing bud, the whole cord was dissected out as completely as possible. It is certain that most of the dorsal portion was removed and that the continuity was

Fig 1

Immediately posterior to union of Limb buds.

Fig 2

Imm. posterior to Fig 1
Section from 7 day (operated) Chick
see text.

W. H. Bates

broken in many places. Finally the tail was cut off, just posterior to the limb bud.

At six days, observation showed the chick to be performing normal movements. The body and legs were moved in a coordinated manner and the movements were periodic, as in the normal chick.

(See Plate 1)

In order to determine the exact condition of the spinal cord, serial sections were made. Their study revealed a continuous spinal cord, but a cord decidedly abnormal and incomplete. For considerable stretches, the nervous tissue of the cord consisted of but a flattened plate which was continuous with the epidermis. (See Plate VI). While nerves which could be traced both to the cord and to the spinal ganglia, were present in the legs, it is hardly conceivable that such a disorganized cord could possibly have sufficed to serve as coordinating mechanism for the normal, coordinated movements which had been observed.

The failure to eliminate movements by operations on the spinal cord, even in such radical operations as in the last two groups described, suggested that the early movements of chick embryos are not coordinated through the central nervous system, but that they are rather, myogenic and independent. While final proof must await the development of a technic which will yield a complete removal of nervous tissue from the posterior half of the embryo, still, the persistence of coordinated movements in chicks in which

the cord is represented for considerable stretches, by a mere flattened band, in which physiological continuity seems out of the question makes it seem highly probable that the above suggestion is correct. Most suggestive in this connection is the finding by M. R. Lewis ('15) that isolated striated muscle fibers, separated from nerves, taken from chick embryos at seven days and grown outside the body in Locke's solution, exhibit rhythmic contractions. It is also interesting that Burrows ('12) has shown that isolated embryonic heart muscle cells beat rhythmically.

Observations on the Earliest Movements in Chick Embryos.

If it be substantiated that the movements in chick embryos are, in early stages, not correlated through the central nervous system,- and the facts just brought out point very strongly in this direction- then a marked difference exists between the early chick and the frog larva. All the studies made by Harrison and his pupil Hooker on frog larvae whether on normal larvae or on larvae deprived of their nerves indicate that the early movements in tadpoles are dependent upon an intact nervous system. Tadpoles from which the spinal cord had been removed were found to perform no spontaneous movements whatever. An analysis of the movements in chick embryos would therefore be most interesting.

Pryor('25) has carried out an extensive series of stimulation experiments on chick embryos, and has found that they do not respond to tactile stimulation until 11 days of incubation, and his results have been confirmed by E. L. and E. R. Clark('14).

Up to the present I have been able to make only a mere beginning at such^{an} analysis, but have already found several points of interest.

First as to the time at which movements start. The age usually given in the atlases, such as the Keibel "Normentafeln", is the early part of the fifth day. I have found movements to begin at 84 to 96 hours in chicks incubated at 38 to 39° C., which is several hours earlier.

Pryor mentions that movements begin on the fourth day. Again, it was found that chicks of 84-96 hours incubation respond to tactile stimulation. When touched with the point of a needle a contraction takes place at the point of application, which is followed by a sigmoid swimming movement of the entire chick, not unlike the normal movement of a chick at this early stage. The response to stimulation was especially noticeable when the stimulus was applied over the myotomes in the wing bud region. In order that tactile responses might not be confused with spontaneous movements, records were taken of the movements of the embryos before, during, and after stimulation. Plate VII shows records of the movements of such embryos, and the increased activity when stimulated.

In order to see whether the conduction of the impulse was carried through the spinal cord, the neural tube was severed, following one of these records, in the region of the omphalomesenteric vessels. Stimulation continued to produce a contraction wave in the myotomes, which continued past the cut in the cord, and which differed in no way from that observed while the cord was intact.

In view of the finding of Pryor, and of E. L. and E. R. Clark, that chick embryos do not respond to tactile stimulation until the 12th day, it is possible that my finding of a response at this very early stage may be explained by the presence at this stage, of a special type of primitive response which is soon lost, and which is replaced at 11 days by a neuro-muscular mechanism. Obviously, further investigation of this problem would lead far beyond the limits of this study.

That the movements were uninfluenced by the transverse section of the cord, furnishes additional evidence for the view that the movements are not coordinated through the spinal cord.

Attempt to Eliminate Movements by Reduction of Temperatures.

Owing to the difficulties which had developed in eliminating movements in the legs by operations on the spinal cord, an attempt was made to devise other methods for producing this result. The first method which suggested itself was to grow chicks at reduced temperatures.

According to Dareste, spontaneous rhythmic movements of chicks cease upon the eggs being cooled to 35 degrees centigrade. E. L. and E. R. Clark obtained similar results. It was thought that, could life be maintained, incubation at subnormal temperatures would afford a simple means of studying the growth of bone in the absence of movement.

Spontaneous Movements and Response to Stimulation in the 90 hour CHICK EMBRYO

Chick 3 days 16½ hrs 6-13-17 6⁰⁰_{pm} length 7.5 mm
6-10-17 1³⁰_{am}

1. Spontaneous Movements of Chick. Time in $\frac{1}{2}$ " + 1"

2. Movements while being Stimulated with needle over myotomes.

Chick 3 days 20 hrs 6-13-17 9²⁵_{pm} length 8 mm
6-10-17 1³⁰_{am}

3. Movements of Chick when stimulated. Stimulation approximately every 1". X = stimulation over region of rt. wing bud.

— few drops Ringer's at room temperature added.
4. Spontaneous Movements.

— returned To incubator for 30'.
5. Spontaneous Movements

6. Movements when Stimulated

Q. J. Zentgraf

Accordingly, eggs, after three and one-half days of incubation at 38-39 degrees Centigrade, were opened for a centimeter area, glass cells attached, and these completely filled with Ringer's solution. A mica cover was then sealed on with wax. Asepsis was observed in all of the procedures. The eggs were placed in a water-jacketed chamber provided with a glass top and a thermometer. The chicks floated high in the solution and were readily observed. The temperature was satisfactorily maintained at 35-34 degrees Centigrade. Upon observing these embryos a twitch, tending to approximate the head and tail, was repeatedly noticed. The thermometer here registered 34 $\frac{1}{2}$ degrees centigrade. To insure accuracy the thermometer was compared with several others. The movement of chick embryos at this temperature coupled with some difficulties caused by the occlusion of blood vessels at the shell margin caused the series to be discarded.

In the following year owing to the persistence of the difficulties in stopping movements by operative procedures, the study of thermal influences was resumed to determine at what temperature movement would cease. An open top paraffine oven fitted with a glass top and a thermometer extending into the warm chamber was used. To get an accurate idea of the movements that might occur and to supplant the use of an assistant, a key, signal magnet and kymograph were used. The latter were obtained through the kindness of Dr. C. W. Greene of the Department of Physiology.

Later in the summer through the courtesy of Drs. C. Judson Herrick and A. J. Carlson in providing material, further studies were made at the Hull Biological Laboratories of the University of Chicago.

The observations consisted, first, in ascertaining that chick embryos do move at temperatures much below 35 degrees Centigrade, and then, in considering what extraneous factors could be responsible for movements at these temperatures. The eggs were incubated at 38-39 degrees Centigrade until the chicks had reached an age sufficient to show vigorous movements- for instance, an age over five days. The shell over the embryo was removed and a few drops of Ringer's solution added to keep the surface of the egg membrane moist. The egg was then placed in a warm chamber to await the drop in temperature. When the egg was at the desired temperature, the kymograph was adjusted and the key depressed during the movements of the chick. Thus the records show the durations of the movements and the periodicity of their recurrence, without any reference to their intensity.

In this manner, chicks were found to move with a fair degree of rhythmicity at 32, 31, $30\frac{1}{2}$, 30, 27 degrees Centigrade, and so on. Only when the temperature was reduced to 20 degrees Centigrade did the movements become so feeble as to be negligible. Even at this temperature a slow slight movement in the right leg was observed. A slight raising of the temperature (to 21 degrees Centigrade) caused vigorous movements to begin. The movements at 21 degrees are less vigorous than those at 35 or 38 degrees Centigrade, but they are present.

The following plate(Plate VII) is practically self explanatory. It is a 'Rectograph' facsimile of a chart compiled by the use of accurate dividers from the kymograph records of movements at four temperatures, all of them below the reported 35 degrees Centigrade. The appended descriptions give in detail the conditions obtaining in each observation.

#1

Egg incubated from 1:30 am	6-7-17	at 101° F.
Changed to incubator 11:00 pm	6-13-17	at 34° C.
Incubator at 5:00pm	6-14-17	at 30.5° C.
Opened at	" "	

Kept moist with Ringer's solution.

Record taken immediately before 8:00 pm 6-14-17

(Note. Irregularities in speed of drum at this time give inequalities in the linear representation of time.)

#2 & #2a.

Egg incubated from 11:00 am	7-6-17	102° F.
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Egg removed 3:30 pm 7-15-17 and an area of shell immediately over the chick was removed and the egg placed in an incubator at 27°C. Two drops of Ringer's solution were used in removing the shell. The thermometer bulb was in contact with the shell through out the observations.

The chick moved continuously in coming to the temperature 27 degrees Centigrade. The temperature was lowered to 26 $\frac{1}{2}$ degrees for an hour before taking the record.

Table reproduced from Kymograph Records (with Dividers)

to show

Periodicity and Duration of Movements
of

Chick Embryos at Reduced Temperatures.
Previous incubation at normal temperature

Duration (only) of movements in relation to time scale
shown by the linear extent of the middle bars.

Immediately before taking, the temperature was raised to 27 degrees, and enough Ringer's solution added to moisten the surface, (the chick was moving at the time of the addition). Except for the two drops noted, no other fluid had been added. The record was taken beginning at 5:30 pm 7-15-17.

#3

Egg incubated from 11:30 am 7-6-17 at 101-102° F.
to 11:30 am 7-17-17 when it was removed, a portion of the shell removed, and then placed in a reduced temperature incubator. At 12:15 a small amount of Ringer's was added. The record was begun at 2:30 pm 7-17-17. The thermometer in contact with the shell registered 23° Centigrade.

#4

Egg incubated from 11:30 am. 7-6-17 at 101-102 $\frac{1}{2}$ degrees F. Same chick as in #3. This egg was cooled by placing in a draft of air. The thermometer was in the egg.

I, II, V & VI were very feeble involving one limb only.

III, VII, VIII & IX involved both limbs.

VIII also involved the body.

Records taken at 7:00 pm, 7-17-17.

At first it was thought that possibly the movements ceased for a time when the temperature was first reduced, to begin after a physiological readjustment had taken place. This was found otherwise by observing a chick continuously until it had reached

the temperature of 27 degrees Centigrade and finding that at no time did the movements cease.

To exclude the possible effect of the Ringer's solution, an 'ooscope' was constructed. This enabled the observation of the movements to be made with an intact shell. Chicks were observed at 30 degrees Centigrade and seen to move, hence air and light also as factors predisposing movement may be ruled out. Examination through the shell is difficult especially under 30 degrees Centigrade because of the decrease in vigor of the movements. When a minimum amount of Ringer's solution was used (See # 2 of Plate VIII) on the opened egg the movements were still found.

A sufficiently large number (in excess of 25) of chicks have been studied to make the observation reliable that 35 degrees Centigrade is not the critical temperature, however the temperature at which movement ceased was determined upon but one chick. Six chicks of different ages were seen to move at 26 degrees Centigrade or below, therefore, the figure 20 degrees Centigrade is considered accurate for the critical temperature.

The possibility that the persistence of movements is due to an increase in the irritability by Ringer's solution, air or light was eliminated. For the lower temperatures, the thermometers were placed in the eggs. The chicks moved periodically, continuously while cooling. Therefore, it was found that:

- 1) Chick embryos are capable of spontaneous periodic movements at any temperature between the normal incubation temperature (38-39 degrees Centigrade) and 20 degrees Centigrade.

- 2) These movements occur at any temperature without a latent period of adaptability.
- 3) The lowness of the temperature at which movements cease makes this method unsuitable for the study of growth of the bones, since growth takes place extremely slowly at this temperature.

Attempts to eliminate movement by the use of chloretone.

Another method which suggested itself for stopping movements was by the use of drugs. The drug which seems most likely to paralyze movements without inhibiting growth processes was "chloretone". This has been used extensively in studies on frog larvae, and it has been found by Harrison ('04) and E. R. Clark ('12) that tadpoles may be immobilized with chloretone for days, without a serious interference with growth processes.

Hoping that this might serve my purpose, it was attempted to immobilize the embryos by using solutions of Chloretone in Ringer's solution. Eggs were half immersed in a 1-1000 solution. Porous plates were sealed on to the egg after a removal of portions of the shell and the egg then semi-submerged. Glass tubes opening into the interior of the eggs and containing Chloretone solution were also tried. These procedures did not eliminate movement. In the summer of 1917, while at the University of Chicago, an effort was made to eliminate movement by the continued application of a Chloretone solution. It was found that 6-10cc. of a 1-1000 solution would greatly impede movement for from 15-20 minutes, at the end of this

time the movements gave evidence of periodicity. Following these results, chloretone was abandoned.

Summary.

While the chief goal of this study- the elimination of movements in chick embryos and its effect on the growth of bone- has not yet been reached, much preliminary work has been done, and facts of interest have developed. The adult skeleton has been studied with particular emphasis on the architecture of the femur and the direction of the forces operating upon it. The technic of selective staining and clearing which will permit an accurate study and comparison of the bones and cartilages, in case the operations are eventually successful, has been adapted to the specific staining properties of chick tissues.

Operations have been performed on chicks of from two and a half to three days, which consisted in (1) transverse section of the cord, (2) removal of short stretches of the cord and (3) attempted removal of the entire posterior portion of the cord. In no case did the operation on the spinal cord affect the movements of the posterior part of the body, during the stages of early bone formation. After simple transverse section regeneration was apparently complete, for the chicks which hatched walked normally. After removal of a section of the cord, however, physiological continuity was not always established, for, in one instance, in such a chick after hatching, voluntary control of the posterior part of the body was absent. Chick embryos were found to respond to tactile

stimuli at a period much earlier than that previously reported. Considering that the nervous system at this age is very rudimentary, this, in addition to the above, seems to justify the conclusion that the early movements of chick are myogenic, not neurogenic, at least that they are not coordinated through the spinal cord.

Chick embryos were found to be very resistant to solutions of chloretone, likewise to low temperatures. The embryos were found to move in a normal manner at a temperature $14\frac{1}{2}^{\circ}$ C. below that previously recorded.

To eliminate movement, then, for the study of bone, it appears that some other means must be devised.

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