ACTIVITY OF CERTAIN ENZYMES IN THE FORELIMB OF THE DEVELOPING CHICK EMBRYO: A HISTOCHEMICAL STUDY

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INTRODUCTION

Histologic and experimental data have, for a number of years, suggested the importance of certain areas or structures within the developing chick forelimb to normal morphogenetic activity. Only recently and to a limited extent has the biochemical analysis of these processes been attempted. Findings to date indicate that certain areas which appear histologically homogenous actually exhibit distinct enzymatic and molecular patterns of development and growth. Either as a result or cause of induction, biochemical activity may increase or decrease, supplying or denying tissues of the limb molecular components which may be necessary to maintain or develop specific structures at the desired time. It is the purpose of this study, therefore, to determine the distribution and development of several important enzymes during critical phases of limb formation in the chick.

By determining the level of enzyme activity, it can be hypothesized that certain biochemical processes

are active in any given tissue. Enzyme histochemistry is one method used to determine this activity. It is true that these techniques introduce problems of interpretation due to the subjective nature of the data and difficulties in diffusibilities of various dyes used. They do, however, enable the investigator to qualitate enzyme activity in individual elements of forelimb tissue and, when differences are sufficiently marked, to make rough quantitative comparisons of this activity. Histochemistry represents the most sensitive enzyme technique available since it can localize activity within a single cell. Biochemical analysis, on the other hand, usually involves homogenation of the entire structure. Obtaining sufficient and pure quantities of any one element becomes almost impossible in non-homogenous structures like the limb bud. For these reasons, the histochemical approach was selected for this study.

The objectives of this investigation are to: (1) survey histochemically a series of enzymes to determine which are active during stage 27, (2) analyze the chick limb bud during stages 19, 21, and 24 for various enzymes found active in the survey, (3) determine histochemically the location and activity

of selected enzymes present during stages 27 and 31 in an attempt to show activity during chondrogenesis and osteogenesis, respectively, and (4) show via hematoxylin and eosin staining of similar tissues any relationships which exist between histologic and biochemical phenomena.

It is hoped that this research will supply information about the molecular activity of forelimb growth and provide an understanding of an induction system of primary importance, setting the stage for future chemical, hormonal, and regeneration studies on the avian limb.

LITERATURE REVIEW

The development of the chick forelimb has been studied for many years and has involved three distinct disciplines: (1) gross and histological, (2) experimental, and (3) biochemical morphogenesis. Saunders (1948) followed by Hamburger and Hamilton (1951) chronologically classified the various stages involved in limb morphogenesis. A comparison of these two systems is illustrated by Table I. The Hamburger and Hamilton method will be used throughout the remainder of this paper.

Zwilling (1961) reviewed in detail the morphological process involved in early limb development. Initial thickening of somatopleure lateral to somites 17-20 called Wolff's crest occurs between stages 15 and 17, followed by a stage of growth and elongation between stages 18 and 23. Stage 18 is essentially that growth occurring as somites 30-36 develop and stage 19 as somites 37-40 develop. During stages 18-20 a distal thickening of ectoderm develops and protrudes

Table I. Comparison between Hamilton and Hamburger (1951), and Saunders' (1948) methods of classifying forelimb development in the chick.

Time in hours	Somites	Hamilton and Hamburger stages	Saunders' stages
50-55	24-27	15	1
51-56	26-28	16	2
52-64	29-32	17	3
65-69	30-36	18	4
68-72	37-40	19	5
70-72	40-43	20	6
84	43-44	21	7
84		22	8-9
84-96		23	10
96		24	
120		27	
168		31	

as a nipple-shaped structure in cross section termed the apical ectodermal ridge (AER). Stage 21 is characterized by the formation of blood vessels and a pronounced ventral bending of the limb bud with rapid elongation of the posterior half. Stage 22 contains a marked mesodermal condensation which will eventually form the limb's skeletomuscular system, and is typified by a large flat paddle-shaped limb directed ventrally and posteriorly. Stage 23 is defined as a further elongation with marked swelling at the base of the forelimb. Romanoff (1960) described the formation of cartilage commencing with stage 27 and ossification with stage 31.

Histologically, Zwilling (1961) showed that two distinct germ layers are involved, the outer ectoderm and the inner mesoderm. After initial formation of the limb bud, rapid proliferation of the mesoderm causes significant elongation and marked ventral bending from stages 18-21. The mesoderm, itself, is spongy and traversed by a number of vessels forming an extensive plexus just under the ectoderm. This plexus is supplied totally by the axial artery as it branches off the most distal portion of the elongated limb bud just under the AER which shunts blood back to the

general circulation via the anterior cardinal vein as reported by Romanoff (1960).

Electron microscopic studies by Jurand (1965) indicate that mesodermal cells during this early stage lack complete cell membranes, and cytoplasmic continuity exists between neighboring cells. They possess an extensive endoplasmic reticulum which appears to be associated with the mitochondria. Golgi bodies are scarce. Jurand found that during this early stage, the ectoderm is a fairly uniform, single layer of cuboidal cells with numerous microvilli along the distal surface and many folds, processes, and microvilli between the cells. In the proximal portion of the bud, the ventral ectoderm is conspicuously thickened and columnar. The cells of the newly formed AER contain numerous elongated mitochondria, many Golgi bodies, and an elaborate endoplasmic reticulum. These organelles are evidently not associated with the high mitotic activity alone, since the mesoblast has a far greater mitotic activity and fewer intracellular structures than the cells of the AER. The basement membrane, an acellular structure approximately 300-400 angstroms thick, exists around the entire bud between the mesoderm and ectoderm.

From stage 21-25, maximal AER development occurs. According to Jurand (1965), the outer and intermediate ridge cells begin to show numerous lysosomes and necrotic centers in the cytoplasm. Degenerating cells appear to be phagocytized by surrounding cells. The basement membrane remains as described previously with the exception of definite discontinuities just under the AER. Romanoff (1960) reported that the mesoderm begins to differentiate slightly with the formation of more distal vessels. Saunders (1966a) noted that a massive zone of necrosis called the posterior necrotic zone (PNZ) appears in the ventral proximal mesoderm and is an essential step in the separation of the upper arm from the body wall. Necrotic centers similar to the PNZ develop within the interdigital tissue and are related to separation of the digits.

Fell (1925) first noted that by stage 27 the central mesenchyme condenses to form long cylinder-shaped structures in a process called chondrogenesis. Precartilage, cells which have not yet taken on the histologic characteristics of cartilage, develop into secreting chondrocytes. Once the cartilaginous structures are formed, a layer of fibroblasts develops around the shaft. This is soon replaced by a layer

of osteoblastic cells in the form of a periosteal collar. Romanoff (1960) reports that the exact time at which these changes occur varies considerably with the different regions of the forelimb.

The phenomenon of a proximal-distal sequence of development is aptly demonstrable in forelimb growth. Through a series of experiments, Saunders (1948) was able to mark various parts of the presumptive forelimb tissue with carbon particles and trace their final destination in the fully developed limb. Very early wing buds were found to contribute to the entire limb, but as elongation proceeded, the apex of the bud first contributed only to the forearm and later only to the extreme distal end of the wing. This same sequence is followed with cartilage, bone, and feather formation indicating conclusively a proximal-distal sequence of limb formation. We can assume from this study that sometime during elongation the cells forming various parts of the adult limb become fully differentiated and determined.

Zwilling (1961) tried to relate the gross and histologic sequence of events with a theory of developmental induction. He hypothesized that initial limb development is dependent upon activation of the

mesoderm by some as yet unexplained mechanism followed by an ectodermal induction by a mesodermal "factor" supplied by the activated mesoderm allowing formation of the AER and the proximal ventral ectodermal thickening. At this stage an interdependence is established between the two layers. Jurand (1965) similarly classified limb development as consisting of three distinct periods. They are: (1) the period of initiation of activity within the somatopleura, (2) the period of interaction between the mesoblast and the epiblast, and (3) the period of formation of definite mesodermal components within the limb mesoblast.

The importance of the ectodermal component cannot be minimized and has been the object of much experimentation. Saunders (1948) reported that carbon particles placed on the AER were found dispersed throughout the entire limb as development proceeded, suggesting that the AER plays a role in the formation of the entire limb rather than one discrete part. If the AER is removed, only a partial limb develops or reduced distal components form from the remaining stump. Bell, et al., (1962) and Goetinck and Abbot (1963) found that if some of the ectodermal cells remained, the limbs proceeded to develop normally.

According to Zwilling (1956a,b), if the ectoderm is rotated 90° leaving the mesoderm untouched, limb outgrowth appears reoriented to the ectodermal component. Transplantation of two AER's to one cut surface of a limb produces partial or complete limb reduplication.

Evidence of an interaction between the mesoderm and ectoderm is probably best supported by studies of the formation of the basement membrane. Speculation by Edds (1958) as to the origin of the membrane led to several series of experiments. Bell, et al., (1959) noted that the basement membrane formed around limb mesoderm implants in the flanks of chick embryos only when covered by a layer of ectoderm. Hay and Revel (1963) injected tritiated proline into Ambystoma larvae and observed autoradiographically that it accumulated first within the mesoderm and ectoderm of the skin and later in the material of the basement membrane, suggesting a possible transfer of products from both layers during the formation of the basement membrane. Proline is found within collagen, a substance existing in large amounts within the basement membrane according to studies by Harkness (1961). Kallman and Grobstein (1965) reported that radioactive proline, when supplied to selected mesenchyme in vitro, passes

through a filter and accumulates along the epithelial side. This material has been called tropocollagen, and is thought to be the soluble monomer of collagen. The epithelium at this point, according to Pierce (1966), catalyzes the polymerization of the tropocollagen into recognizable collagen fibrils. Since presence or absence of the basement membrane may significantly control normal limb development, interactions between these two layers with regard to this important structure need considerably more study.

To support the theory that a mesodermal factor is involved in ectodermal induction, Saunders (1949) placed a small mica plate between the mesoderm and ectoderm. Both AER formation and elongation failed to occur. In 1963, he was able to demonstrate that reversed wing bud stumps, if placed anterioposteriorly and dorsoventrally, will allow for duplicate digit formation, presumably due to the redistribution of the maintenance factor within the postaxial area of the mesoderm. If, however, a small Mylar sheet is placed between the reoriented stump and the postaxial mesoderm, reduplication fails to occur. This evidence suggests that postaxial tissues supply the necessary factor for normal morphogenesis. Zwilling (1964)

supported the conclusion that an interaction does occur, for when limb ectoderm or mesoderm is grown alone, or when limb mesoderm is grown with non-limb ectoderm, only random, unrecognizable structures develop. Limb mesoderm and limb ectoderm together, however, form recognizable limb structures with some exhibiting marked digit formation.

Biochemically, relatively little has been attempted to elucidate the mechanisms involved in limb development and growth. Milaire (1962), Moog (1944), and Jurand (1965) noted the histologic development of acid phosphatase (AcP) activity in limbs of several vertebrates including the chick. During stages 18-22, Milaire observed AcP activity in the ventral ectoderm and AER. Ultrastructurally, Jurand noted that the lysosomes, extra-nuclear necrotic centers, and Golgi bodies within the AER demonstrated high activity during stage 24. Moog found that the skin lost most of its activity by stage 29, after which time all layers became negative except for slight reactivity in the nuclei. Mesenchyme generally retained its activity until differentiation occurred, and cartilage, when it first appeared, was negative. Alkaline phosphatase activity was also studied histochemically by Milaire

(1962) and Moog (1944) and biochemically by McWhinnie and Saunders (1966). Alkaline phosphatase activity appeared strongly in the AER, weak in the ventral ectoderm, and non-existent in the dorsal ectoderm in most vertebrates during stages 18-22. By stage 29, the activity of the ectoderm was lost, while mesenchymal activity remained until differentiation, disappearing in developing cartilage.

Milaire (1962) observed ribonucleic acid (RNA) in the proximal postaxial ectoderm during stages 18-22. The ventral mesoderm exhibited high RNA content just under the ventral ectoderm. Hinrichsen (1956) noted that the highest concentration existed in the AER.

Weel (1948) reported that ascorbic acid accumulated as a ring around the base of the limb bud from stage 24-27, disappearing as development proceeded. It was found to collect between the developing digits and in myogenic areas prior to histologic differentiation. He also found that fat accumulated in higher concentrations in mesenchymal cells than in chondroblasts.

Weel (1948), Prichard (1952), and Milaire (1962) studied glycogen accumulation in the developing forelimb. Weel observed glycogen only in muscle after histologic differentiation. Milaire noted that it was

present in preosteoblasts and cartilage, but negligible in periosteal fibroblasts. All of this activity substantiates our previous knowledge that an interaction exists between the ectoderm and mesoderm, especially in the ventral and distal areas of the elongated bud.

MATERIAL AND METHODS

Preliminary Histochemical Survey

Fifteen enzymatic systems were studied in an attempt to determine which of these enzymes were active within the limb bud of a stage 27 embryo. From these data the most informative enzymes were selected and studied in detail over longer periods of limb development. Fertilized eggs from White Leghorn chickens were incubated at 100°F for approximately 5 days. Five limbs were obtained for each study, prepared and sectioned at 12 microns, and stained according to the selected histochemical technique. A known positive rat tissue, usually liver or kidney, was always included in each staining procedure serving as the false negative. In addition, identical tissue was processed in incubation media which contained no substrate; this served as the false positive control.

The following is a list of the enzymes studied and the techniques used:

1. Acid phosphatase - simultaneous coupling azo dye technique. (Barka, 1960)

2. Acetylcholinesterase - acetylthiocholine method. (Gomori, 1952)

3. Adenosine triphosphatase - lead nitrate method. (Wachstein and Meisel, 1957)

4. Aldolase - tetrazolium salt method. (Abe and Shimiger, 1964)

5. Alkaline phosphatase - simultaneous coupling azo dye technique. (Burstone, 1958)

6. Cytochrome oxidase - Burstone technique. (Burstone, 1959)

7. DPN-diaphorase - tetrazolium salt technique. (Scarpelli, et al., 1958)

8. Glucose-6-phosphate dehydrogenase - nitro-BT method. (Nachlas, et al., 1958)

9. β-glucuronidase - naphthol AB-BI glucuronidide method. (Hayashi, et al., 1964)

10. Lactic dehydrogenase - nitro-BT method. (Nachlas, et al., 1958)

11. Leucine aminopeptidase - Nachlas technique. (Nachlas, et al., 1957a)

12. Malic dehydrogenase - nitro-BT method. (Nachlas, et al., 1958)

13. Monamine oxidase - tetrazolium salt technique. (Glenner, et al., 1957)

14. Succinic dehydrogenase- nitro-BT method. (Nachlas, et al, 1957b)

15. TPN-diaphorase - tetrazolium salt technique. (Scarpelli, et al., 1958)

The results indicated that three enzymes exhibit moderate to intense activity at this stage of development. These were: (1) acid phosphatase (AcP), (2) adenosine triphosphatase (ATPase), and (3) lactic dehydrogenase (LDH). In addition, the two diaphorase systems, DPN-d and TPN-d, were found to be strongly positive. Enzymes found to give weak positive reactions included alkaline phosphatase, leucine aminopeptidase, monamine oxidase, and aldolase. Enzyme activity was found negative for succinic dehydrogenase, malic dehydrogenase, cytochrome oxidase, glucose-6-phosphate dehydrogenase, β-glucuronidase, and acetylcholinesterase.

AcP, ATPase, and LDH Activities in Forelimb Development

Three enzymes exhibiting moderate to intense activities in the preliminary survey were examined further in forelimb buds from stage 19, 21, 24, 27, and 31 embryos. These enzymes were AcP, ATPase, and LDH. White Leghorn chicken eggs were obtained and incubated routinely at 100°F for the desired period of time. The stage of embryonic development was determined from gross examination of the embryo and histological analysis of forelimb structures as outlined by

Hamburger and Hamilton (1951). At least 20 limbs were examined for each stage selected. Serial sections were prepared.

AcP activity was studied using Barka's (1960) simultaneous azo dye technique. Limbs were obtained and placed for approximately 12 hours in a cold 4% neutral formol calcium solution and then transferred to a .88M sucrose solution. Tissues were frozen and sectioned on a cryostat at 12 microns. Rat kidney sections served as false negative and positive controls. Within each embryonic section, the liver and spinal cord always gave intense positive reactions.

ATPase activity was examined using Wachstein and Meisel's (1957) lead nitrate method. The method requires fresh frozen sections. All tissues were once again cut at 12 microns, incubated and stained as required. Rat liver served as false negative and positive controls. Within each section of limb and associated body structures was found some liver tissue which always exhibited an intensely positive reaction and served as an intraembryonic control.

LDH was studied further using the Nachlas, et al., (1958) nitro-BT method, which also required fresh frozen tissue. During each staining trial or

run, a rat kidney section served as the false negative and positive control. Liver again acted as an intraembryonic control since it always stained intensely for LDH. Since the final transfer of electrons in the conversion of lactate to pyruvate is dependent upon the functional state of the DPN and TPN diaphorase systems, and since our tetrazolium salt technique also depends on these systems to form the final colored precipitate, it was necessary to determine the activity of the two diaphorases. Using the Scarpelli, et al., (1958) technique, strong activity was found to exist from day 3 on, and would not, therefore, interfere with the LDH determinations.

In an effort to compare normal histologic structures with enzymatic activity present in limb morphogenesis, every tissue stained histochemically was also stained with hematoxylin and eosin.

RESULTS

Enzyme activity was noted according to the following empirical scale: 0, negative; 1, slight; 2, moderate; and 3, intense. Tables II, III, and IV illustrate average histochemical values observed in the various structures of the forelimb. The results were statistically analyzed according to the Mann-Whitney two-tailed U test (Siegel, 1956). Figures 1-5 represent hematoxylin and eosin stained sections of normal forelimb bud from stage 19, 21, 24, 27, and 31 embryos.

Acid Phosphatase

During stage 19, the limb bud exhibited a 1.0-1.1 activity throughout the ectoderm and mesoderm. However, it was observed that slightly more activity persisted in the newly-formed AER (Fig. 6). By stage 21, the AER developed a 2.4 activity concentrated within a central core and bordering on the underlying mesoderm (Figs. 8 and 9). The axillary

ectoderm exhibited a significantly increased 1.9 activity (Fig. 7). All other areas of the mesoderm and ectoderm retained a low 1.0-1.3 activity (Fig. 7). By stage 24, the AcP activity of the AER developed into two or more 3.0 sites of activity. One was located in the central core as described in the previous stage, while the others were found at the extreme tip of the AER bordering on the amnionic cavity (Fig. 10). The activity of the axillary region decreased considerably by this stage, while the rest of the ventral ectoderm maintained its 1.9 activity (Fig. 11).

By stage 27, the AcP activity of the ectoderm around the entire limb increased markedly giving a 2.6-2.7 reaction. Enzyme activity within a majority of cells observed was localized along the apical portion of these ectodermal cells due primarily to displacement of the cytoplasm by the nuclei which line the basal border (Fig. 14). The ectoderm which previously represented the AER still exhibited a 3.0 activity, but decreased considerably in size to approximately one or two cells thick (Fig. 13). The mesoderm still gave a low 1.5 activity with the exception of an almost total lack of activity in those cells condensing to form cartilage (Fig. 12).

The relatively intense AcP activity noted in the ectoderm during stage 27 remained high by stage 31, but gave some indication of a slight decrease in activity. Although not significantly different from the previous stage, the differentiating mesoderm exhibited a slight increase in activity. The cartilage gave a markedly increased 1.6 activity. Moderate 2.2 activity was observed within the periosteal collar around the cartilage (Fig. 15).

Adenosine Triphosphatase

During stage 19, a 1.0 ATPase activity appeared evenly distributed throughout the entire limb bud (Fig. 16). The first sign of a significant increase in activity was seen in the ventral half of the mesoderm in stage 21 embryos (Fig. 17). By stage 24, the ventral half of the mesoderm maintained this increased activity, while the cells of the AER tended to show a slight increase in activity. All other structures demonstrated only slight 1.0 activity (Fig. 18). The activity in the ventral mesoderm tapered off within the ventral body wall near the axilla (Fig. 18).

The enzyme activity during stage 27 varied considerably from that of stage 24. The AER, itself, now

disappeared, and the ectoderm only exhibited a slight 1.0 activity. The newly-formed condensation of cartilaginous cells indicated a moderate 2.0 activity. The ventral mesenchyme maintained its moderate activity. A distal reactive center of mesenchyme gave 2.0 reactivity (Fig. 19).

By stage 31, the entire mesoderm exhibited a 1.8 activity with the exception of a 2.8 reaction in the cartilaginous cells and 3.0 activity within the periosteal collar. The activity surrounding the cartilage tapered off on both ends forming a cylinder around the midshaft region. The ectoderm retained its 1.0 activity (Figs. 20 and 21).

Lactic Dehydrogenase

A 1.0 LDH activity was evenly distributed throughout the limb bud during stage 19 (Fig. 22). By stage 21, a significantly increased 1.8 activity was seen in the area of the AER and in the ventral mesodermal condensation (Fig. 23). During stage 24, 1.6 activity remained in the AER (Fig. 23). The ventral mesoderm exhibited similar 2.2 activity which extended into the body cavity as two branches. One followed the axillary area into the mesoderm of the ventral body

wall, while the other extended upward into the mesoderm of the dorsal body wall and into the mesoderm of the limb, itself. Intense 3.0 activity localized in a single row of mesodermal cells just under the ectodermal layer along the ventral mesodermal condensation (Fig. 25). Another site of intense 3.0 activity was observable in the mesodermal cells lining the ventral surface of the AER (Fig. 24).

The mesodermal activity during stage 27 was similar to the above. The intense 3.0 activity in the row of mesodermal cells along the ventral ectoderm was still present (Fig. 27). The more moderate 2.1 activity of the ventral mesodermal condensation extended into the ventral body cavity and appeared distinct from the 1.0 activity within the dorsal proximal mesoderm (Fig. 26). The cartilage stained moderately. The ectoderm during this stage of development exhibited only a 1.1-1.2 activity.

By stage 31, the activity within the ectoderm increased significantly to 2.0 around the entire limb. The activity of the dorsal mesoderm increased to 1.6, while the ventral mesoderm maintained its already increased 1.8 activity. The cartilage cells indicated

an intense 3.0 reaction (Fig. 28). Only a slight .9 reaction could be seen surrounding the cartilage in the cells of the periosteal collar (Fig. 29).

Table II. Acid phosphatase activity in developing chick forelimb.

Stage	Ectoderm			Mesoderm			
	DE	VE	AER	DM	VM	C	B
19	1.0	1.1	1.1	1.0	1.0	-	-
21	1.3	1.9**	2.4***	1.0	1.1	-	-
24	1.3	1.9	3.0**	1.0	1.1	-	-
27	2.6***	2.7***	-	1.5	1.5	.2	-
31	2.3	2.3	-	1.9	1.9	1.6***	2.2

DE Dorsal ectoderm
VE Ventral ectoderm
AER Apical ectodermal ridge
DM Dorsal mesoderm
VM Ventral mesoderm
C Cartilage
B Bone

0 Negative
1.0 Slight activity
2.0 Moderate activity
3.0 Intense activity

* $P(u \leq x) = .05$ as compared to the previous stage[1]
** $P(u \leq x) = .02$ as compared to the previous stage[1]
*** $P(u \leq x) = .002$ as compared to the previous stage[1]

[1]Mann-Whitney U Test; Two-tailed Test (Siegel, 1956)

Table III. Adenosine triphosphatase activity in developing chick forelimb.

Stage	Ectoderm			Mesoderm			
	DE	VE	AER	DM	VM	C	B
19	1.0	1.0	1.0	1.0	1.0	-	-
21	1.0	1.0	1.3	1.0	1.8 **	-	-
24	1.0	1.0	1.8	1.0	2.0	-	-
27	1.0	1.0	-	1.0	1.6	2.0	-
31	1.0	1.0	-	1.8 **	1.8	2.8 **	3.0

DE Dorsal ectoderm
VE Ventral ectoderm
AER Apical ectodermal ridge
DM Dorsal mesoderm
VM Ventral mesoderm
C Cartilage
B Bone

0 Negative
1.0 Slight activity
2.0 Moderate activity
3.0 Intense activity

* $P(u \leq x) = .05$ as compared to the previous stage[1]
** $P(u \leq x) = .02$ as compared to the previous stage[1]
*** $P(u \leq x) = .002$ as compared to the previous stage[1]

[1]Mann-Whitney U Test; Two-tailed Test (Siegel, 1956)

Table IV. Lactic dehydrogenase activity in developing chick forelimb.

Stage	Ectoderm			Mesoderm			
	DE	VE	AER	DM	VM	C	B
19	1.0	1.0	1.0	1.0	1.0	-	-
21	1.0	1.0	1.8***	1.0	1.8**	-	-
24	1.0	1.2	1.6	1.2	2.2	-	-
27	1.2	1.1	-	1.0	2.1	2.0	-
31	2.0***	2.0***	-	1.6**	1.8	3.0***	.9

DE Dorsal ectoderm
VE Ventral ectoderm
AER Apical ectodermal ridge
DM Dorsal mesoderm
VM Ventral mesoderm
C Cartilage
B Bone

0 Negative
1.0 Slight activity
2.0 Moderate activity
3.0 Intense activity

* $P(u \leq x) = .05$ as compared to the previous stage[1]
** $P(u \leq x) = .02$ as compared to the previous stage[1]
*** $P(u \leq x) = .002$ as compared to the previous stage[1]

[1]Mann-Whitney U Test; Two-tailed Test (Siegel, 1956)

DISCUSSION AND CONCLUSIONS

Functions and Distribution of AcP, ATPase, and LDH

AcP is believed to be concerned with the hydrolysis of phosphoric acid esters and, in particular, phosphomonesters. According to Schmidt and Laskowski (1961), this results in the formation of an orthophosphate. Schmidt (1961) noted that AcP is active in areas of high cellular activity such as in the cells of the prostate gland, liver, kidney, and mucosa of the small intestine. Brachet (1960), Cone and Eschenberg (1966), and Clever (1966) reported high activity within cells undergoing rapid degradation. AcP appears to be concentrated in the Golgi bodies (Jurand, 1965; Sobel and Avrin, 1965), lysosomes (Jurand, 1965; Brachet, 1960; Clever, 1966), and cytolysomes (Jurand, 1965). In addition, the nuclei and cell membranes of some cells show moderate activity (Jurand, 1965). AcP is not an homogenous enzyme according to Pearse (1960), but made up of many different ones which share a similar pH optimum of approximately 5.0. Differentiation

of these specific enzymes requires biochemical analysis of pH activity curves, quantitative response to various substrates, and inactivation or suppression by selected inhibitors (Schmidt, 1961).

Kielley (1961) described ATPase as an essential enzyme in the release of energy from adenosine triphosphate. It is found in all living cells, and, in general, exists in association with formed intracellular structures. Several different types of enzymes exist. One is located within the structure of the myofibrils of myosin and is presumably related to muscular contraction. Others are found associated with the endoplasmic reticulum, cell membranes, and mitochondria (Essner, et al., 1965; Kielley, 1961). Moog (1947) and Robinson (1952) analyzed the content of ATPase in developing chick muscle. The enzyme activity was found to precede histologic and functional differentiation. In early developing cells, the sarcoplasmic elements appeared to be highest in activity. Later, this activity diminished and was replaced by a high content of myofibrillar ATPase. In developing rat muscle, Herrman and Nicholas (1948) and de Villafranca (1954) showed that activity increased with functional differentiation and contractility.

According to Barka and Anderson (1963), LDH catalyzes the reversible conversion of pyruvate to lactate. Its presence indicates activity of the glycolytic cycle in the production of lactate from glucose, and is found in all animal tissues, but mostly in muscle and liver cells (Schwert and Winer, 1961). Lindsay (1963) described five isozymic structures of LDH. Unfortunately, they cannot be distinguished via standard histochemical procedures.

Possible Implications of Enzymatic Activity

Enzymes during Jurand's "period of initiation" exhibit only slight ubiquitous activities. The absence of pattern reflects the absence of differentiated tissue. The slight activity of the enzymes fails, however, to correlate with an increase in the proliferation rate of either the mesoderm or ectoderm, which may seem somewhat unusual in view of the high energy requirements of embryonic tissues.

Starting with stage 21, a series of structures begin to differentiate from the original germ layers. This change is reflected by enzymatic activity prior to and during histologic differentiation. One of the first recognizable structures is the ventral mesodermal

condensation in the postaxial mesoderm. Its rapid mitotic rate and high RNA content bear a possible relationship to its inductive capacity as described by Saunders and Gasseling (1963). The present study indicates that moderate ATPase and LDH activity reflect this possible initiation of differentiation as the cells begin to utilize energy via the glycolytic cycle. It is precisely at this time that the ectoderm begins to thicken in the formation of the AER in response to an inductive activity which is unique to the mesoderm of the limb bud. Whether or not this or any other biochemical activity is absent in non-limb mesoderm has yet to be studied.

The AER deserves particular attention because of its almost certain role in the distal outgrowth of the normal forelimb. It appears histologically by stage 18, reaching maximum development between stages 22 and 26. Any interaction between the AER and the underlying mesoderm would most certainly occur at this time, for during stage 27, a rapid series of intracellular changes occur in the ridge, both structurally and biochemically, which lead to its complete destruction.

Numerous previously reported experimental procedures attest to the importance of stages 22-26. For

example, carbon, placed within the limb mesoderm prior to stage 21, could not be located by Saunders (1948) in the newly formed digits. Searls (1965) noted that radioactive sulfate uptake by precartilage cells begins during stage 22 and not before. According to Saunders, Cairns, and Gasseling (1957), leg mesoderm from embryos in stages 24 and 25, when grafted into proximal levels of forelimb mesoderm, forms toes, scales, and claws typical of hindlimb development, while earlier mesodermal grafts do not. Saunders and Gasseling (1957) showed that by stage 24 the properties of the prospective humeral feather tracts are fixed and resist changes following the reorientation of presumptive component mesoderm and ectoderm. Stage 26 hind limb mesoderm loses its ability to form bony elements if inserted under the AER of the forelimb (Saunders, et al., 1959). Finally, Searls (1965) found that radioactive sulfate disappeared from the basement membrane and was no longer absorbed by the mesoderm by stage 26. All of these studies tend to support the hypothesis that inductive interaction begins on or around stage 21, and is progressively completed in the various elements of the forelimb by stage 26.

Balinsky (1956) and Goetinck (1966) suggested

that the presence of the basement membrane between the mesoderm and ectoderm during this important period prevents any possible interaction, while its absence allows for an interaction between cells of different basic type. Balinsky (1956, 1957) noted that as supernumary limbs are induced in amphibians, the basement membrane is reduced or absent. Also, if the amputation surface of a severed amphibian limb is covered with epidermis and dermis, it fails to regenerate (Balinsky, 1956). Grobstein (1962) showed that mesenchyme in vitro reacts with pancreatic rudiments to form recognizable acini across a Millipore filter as long as the pore size was large enough for certain molecules to pass through and induce adjacent tissues.

Pierce (1966) observed that the basement membrane may act in a similar way and filter out many of the undesirable molecules. Finally, Goetinck and Abbot (1963) were able to show that ectodermless limb buds of the chick, if exposed to collagenase and then recombined with the ectoderm, form outgrowths if implanted into the flank area. Since this treatment results in destruction of the basement membrane, it can be said that the membrane is not essential for limb outgrowth to occur.

Jurand (1965) offered visual evidence that direct

contact occurs between the AER and the mesoderm. From stage 22-26, "discrete discontinuities" were observed in the basement membrane at the base of the AER. The study presented here suggests possible biochemical events which precede and coincide with this phenomenon. Moderate or intense AcP activity from stage 21-24 may indicate destruction of the membrane, since this enzyme is often released from lysosomes with accompanying proteolytic agents (Tappel, et al., 1963). The intense LDH activity observable in the mesoderm under the AER during stage 24 suggests rapid glycolytic activity, in an attempt, perhaps, to rebuild the membrane, utilize products of the destructive process, and/or participate directly or indirectly in an interaction between the AER and the mesoderm. It would be unwise to speculate further or more specifically on this point considering the sparse data available from this study alone.

Non-ridge ectoderm during this phase of development gave practically no evidence of the underlying activity within the mesoderm with the exception of moderate AcP activity in the ventral ectoderm as observed by Moog (1944) and later verified by this study. A peculiar single layer of intense LDH activity within the mesoderm is difficult to explain except as a

reflection of the active mesodermal tissues below.

Data suggest two essentially different processes at work within the AER. One is the process characterized by cells undergoing rapid cellular activity. It is exemplified by the presence of numerous elongated mitochondria (Jurand, 1965), frequent endoplasmic reticulum (Jurand, 1965), and high RNA concentration (Hinrichson, 1966). The study presented here reveals, in addition, high amounts of ATPase and LDH activity within the ridge. These changes are consistent with rapid protein synthesis and accompanying cytodifferentiation and inductive capacities occurring from stage 21-26. The second process is that of cellular destruction and autolysis. By stage 23, the appearance of numerous lysosomes, Golgi bodies, and necrotic centers called "cytolysomes" precedes this phenomenon. Within a short time these structures begin to break down, releasing large quantities of proteolytic enzymes into the cytoplasm and thus, destroying the cell. AcP, the enzyme found in highest concentrations within the lysosomes, seems to reflect more than any other entity the destruction of these cells culminating in the disappearance of the AER by stage 27. The data presented here vividly depict the AcP activity localized in the ridge

during this phase of development.

Electron micrographs of these cells by Jurand (1965) indicate that these changes originate within the cytoplasm of the cells and only involve the nucleus secondarily. Moreover, the initiation of this destruction appears to be spontaneous and originates from within the cells themselves. This process is similar to the destruction of other necrotic centers of the forelimb as described by Saunders (1966a). The role of genetic or hormonal factors in this phenomenon have yet to be determined.

In this study, the PNZ could not be demonstrated as possessing high AcP activity. Saunders (1966a) has, however, been able to demonstrate activity within these cells. During stage 27, a distal necrotic center exhibited moderate ATPase activity, indicating high energy utilization needed for digit formation.

Indications suggest that muscle begins to differentiate as early as day 4. Moscona and Moscona (1952) showed that isolated stage 24 myogenic and chondrogenic cells when grown in tissue culture developed along divergent paths. Spontaneous movement was observed to originate on day 7 (Balaban, 1967). Although some ATPase and LDH activity preceded

functional differentiation, the most significant increase was noted in the dorsal mesoderm at precisely the time that movement was first observed, according to the present study. In addition, AcP activity showed some signs of increasing. The results suggest that enzymatic activity in the dorsal mesoderm, at least, coincides with functional differentiation.

Histologically, the first suggestion of cartilage formation occurs at approximately stage 27. However, it appears that cells are biochemically unique as long as three days before histologic differentiation. Radioactive sulfate was first detected by Amprino (1955) in cells during stage 24. Later, Searls (1965) found that precartilage accumulated sulfate as early as stage 22. Deuchar (1963) noted that proline, a precursor of collagen, was observed in the perichondrial layer before collagen became visible histologically in connective tissue using stains such as Malloy's and Mason's procedures or by standard biochemical tests. Similarly, developing cartilage in Ambystoma limbs undergoing regeneration, accumulated proline readily according to Revel and Hay (1963). In the study presented here, Moog's (1944) results were confirmed in that AcP activity noticeably disappeared from

differentiating cartilage, leading to the assumption that this enzyme does not play a role in chondrogenesis. Likewise, no evidence can be found to suggest that ATPase or LDH were involved.

Once the cartilaginous cells differentiated, an entirely different picture appeared. All enzymes studied became moderately to intensely active. The only explanation that seems plausible is that once cartilage is formed, it immediately begins the process of decay and degeneration as described by Fell (1925). Rapid cellular growth and chemical synthesis may occur in these cells just prior to destructive processes as discussed previously in the AER. The rapid proliferation may, instead of being a separate entity, bring about or initiate the destruction which follows. The two may in certain embryological situations be inseparably linked. The cells may, in a sense, "burn themselves out."

Bone formation in stage 31 embryos is most readily studied within the cells of the periosteal collar. Pritchard (1952) discovered that glycogen exists in these cells and is presumably hydrolyzed by the phosphatases, accounting for the increase in AcP activity observed in the present study. This process liberates

numerous free phosphate ions, which are now capable of combining with calcium in the formation of an inorganic matrix during the process of osteogenesis. Energy requirements would appear to be immense as depicted by the intense ATPase reactivity of the cells.

As the epidermis develops, AcP activity increases uniformly, levels off during stage 27, and then begins to decline. This same phenomenon was observed earlier by Moog (1944). The results of the study presented here indicate that LDH activity increased significantly during stage 31. The reason for this activity during these stages remains undetermined at this time.

General Considerations

The above study has involved primarily changes in biochemical patterns, both spatially within different tissues at a given time, and temporally between similar structures at progressively increasing intervals. As far as possible, the data presented here and elsewhere link gross morphological, histologic, and electron microscopic data with enzymatic activity occurring as structures appear and develop. In certain instances, disappearance of structures has been correlated with decreased enzyme activity; i.e. disappearance of the

AER coincides with the disappearance of AcP activity within the ridge cells and with LDH activity in the mesoderm just under the ridge. Ultimately, of course, the biochemical changes which occur prior to and during cytological differentiation should be determined. Unfortunately, in this study, only a few of these changes could be demonstrated.

Histochemical procedures, such as those used here, demonstrate cellular localization of enzyme activity within the limits of the techniques selected. The results are subject to problems of diffusion, errors in quantitation, and losses in enzyme activity between sectioning and incubation of tissues. Although somewhat academic, it is not known if increases within any particular tissue or structure come from increased production of the enzyme which is probably genetically controlled, or from an increase in the activation of preformed enzymes as controlled by the cytoplasm or the environment in general. The techniques used were developed initially for laboratory animals, such as rats and mice, and for adult tissues which react somewhat differently than embryonic limb buds. Also, there is little evidence to support the fact that an enzyme found within adult tissues plays

any role in early limb morphogenesis that cannot be supplied by completely different molecular structures. Finally, staining of enzyme activity involves placing the tissue in optimal temperatures, supplying necessary coenzymes and cofactors, and allowing them to incubate for the proper length of time, a process which establishes an environment much different than the conditions found within the normal developing limb. It has often been said that results are only as good as the methods used. Unfortunately, these are the best techniques available at the moment and their shortcomings should be realized.

Many of the enzymes studied exhibited only slight activities. Small residual levels of activity may actually be very important to the maintenance and growth of cells during these phases of development. It may be that they are simply being manufactured and stored in a latent form waiting to be triggered by the internal or external environment of the cell. A second possibility is that the cellular enzymes may not play any part in metabolism until a certain threshold is reached. The exact role of this early activity is not known.

Future studies should involve the use of radioactively-labeled molecules that form necessary

components of various substrates, macromolecules, and enzymes. Accumulation of these structures are, in fact, more likely to occur before enzyme activity can be observed. An excellent example of this phenomenon is the induction of specific enzymes by their substrates, a process whereby external or internal molecular regulators "turn on" the genetic apparatus controlling specific enzyme production (Waddington, 1966). Histochemistry and electron microscopy will be used together by future developmental scientists to demonstrate enzyme activity within the smallest organelles. Ultimately, however, biochemical embryology will be concerned with the normal as it compares to the experimental variable. For example, by supplying selected inhibitors or limiting the amount of available cofactors, enzyme activity in a tissue or structure can be controlled or inhibited and the effect studied. Experiments concerned with interactions between the two germ layers could involve the use of various size Millipore filters and mica or Mylar sheets placed between the cells. Fractionalization and analysis of limb mesoderm and ectoderm may result in the purification of the hypothesized mesodermal or ectodermal "factors" which could be studied

biochemically and histochemically *in vivo* and *in vitro*. All these things and more are needed to elucidate the problems of forelimb morphogenesis in the chick.

SUMMARY

The development of acid phosphatase, adenosine triphosphatase, and lactic dehydrogenase activity was studied histochemically in the chick forelimb from stage 19 through 31 (ca. day 3 to day 7). Development of the apical ectodermal ridge, mesenchyme, non-ridge ectoderm, muscle, cartilage and bone was observed. Acid phophatase activity was highest in the apical ectodermal ridge, epidermis, and periosteal collar surrounding the developing long bones. Adenosine triphosphatase activity was found intensely only within the cells of the periosteal collar and cartilage. Lactic dehydrogenase activity was concentrated mainly in mesodermal components such as the ventral mesodermal condensation, the row of mesodermal cells immediately under the ventral ectoderm, and in the cartilage. The appearance of enzyme activity reflected the differentiation of the mesoderm and ectoderm as the various structures and tissues form. The hypothesized interaction between the

mesoderm and ectoderm was particularly evident in activities located within the AER, the row of ventral mesodermal cells, and ventral ectoderm.

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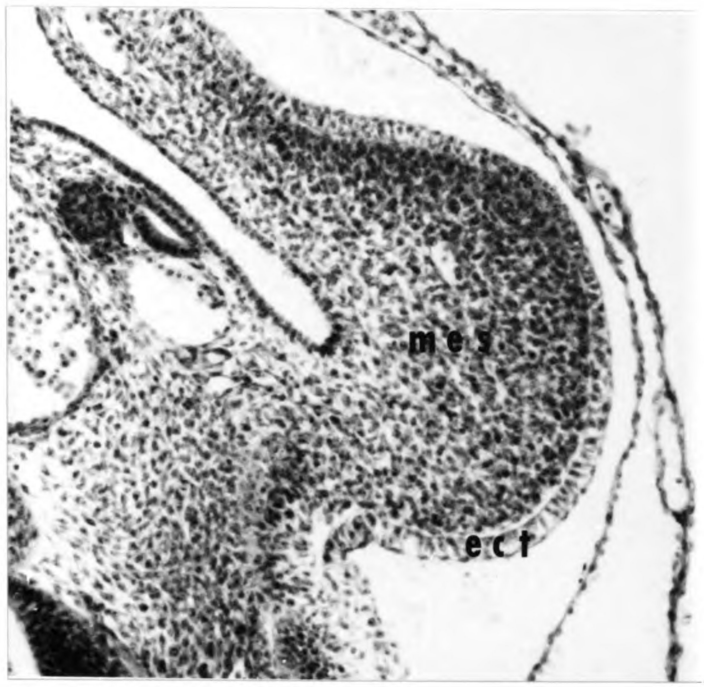
Figure 1. Forelimb bud. Stage 19. Note the mesoderm (mes) and ectoderm (ect) of the newly-formed limb bud. H. & E. X140.

Figure 2. Forelimb bud. Stage 21. The ectoderm is composed of the apical ectodermal ridge (AER), the dorsal ectoderm (de), and the ventral ectoderm (ve). The ectoderm of the limb is continuous with that of the amnionic sac (as). Many blood vessels (bv) appear. The mesoderm has undergone marked condensation. H. & E. X140.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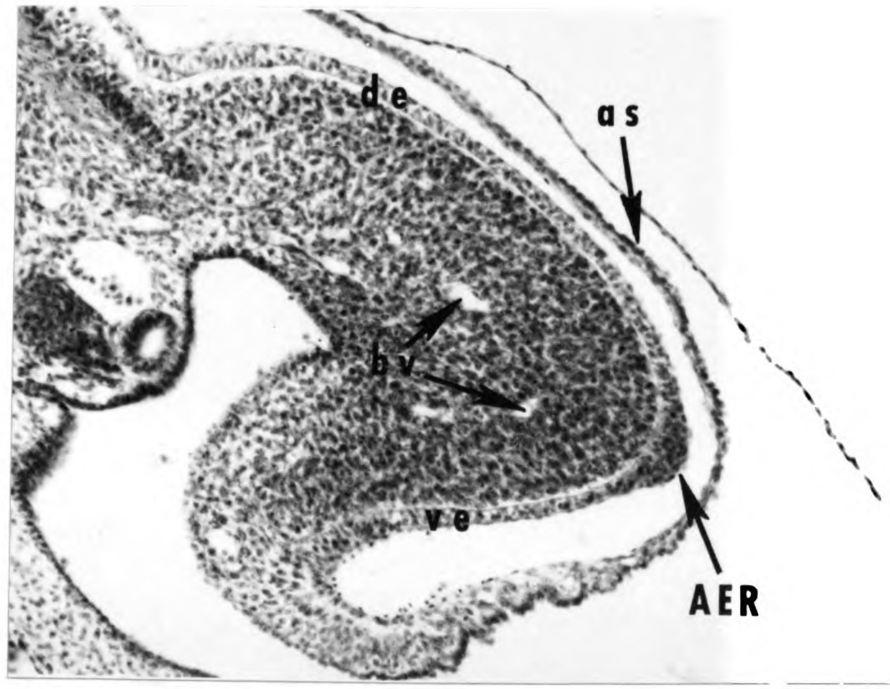
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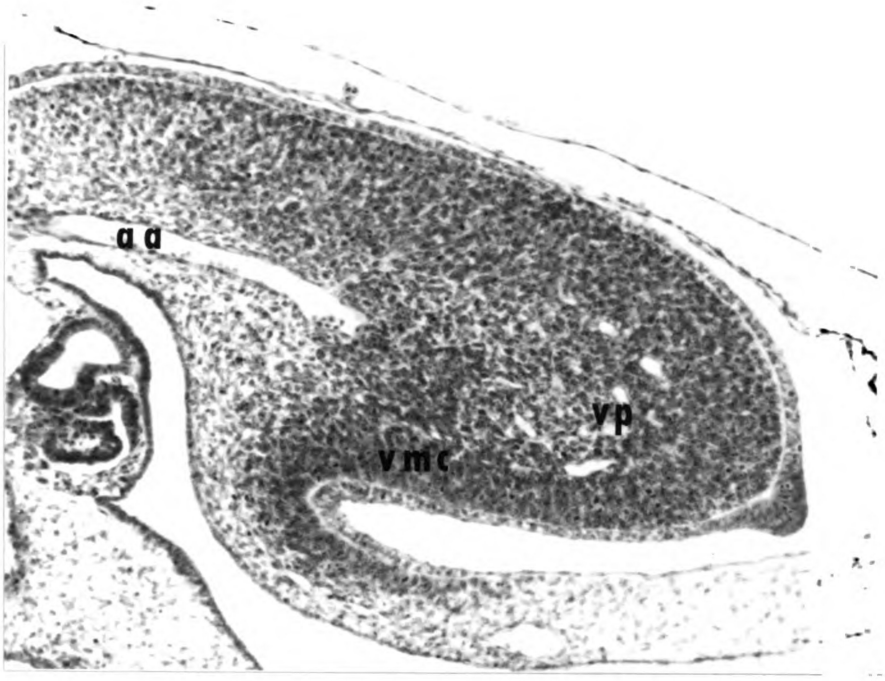
Figure 3. Forelimb bud. Stage 24. This stage is characterized by further elongation of the bud, a ventral mesodermal condensation (vmc), a large axillary artery (aa), and a venous plexus (vp) just under the AER. H. & E. X140.

Figure 4. Forelimb. Stage 27. Mesenchyme has condensed to form cartilage (cart). Numerous arteries (a) and nerves (n) are seen entering the limb. H. & E. X46.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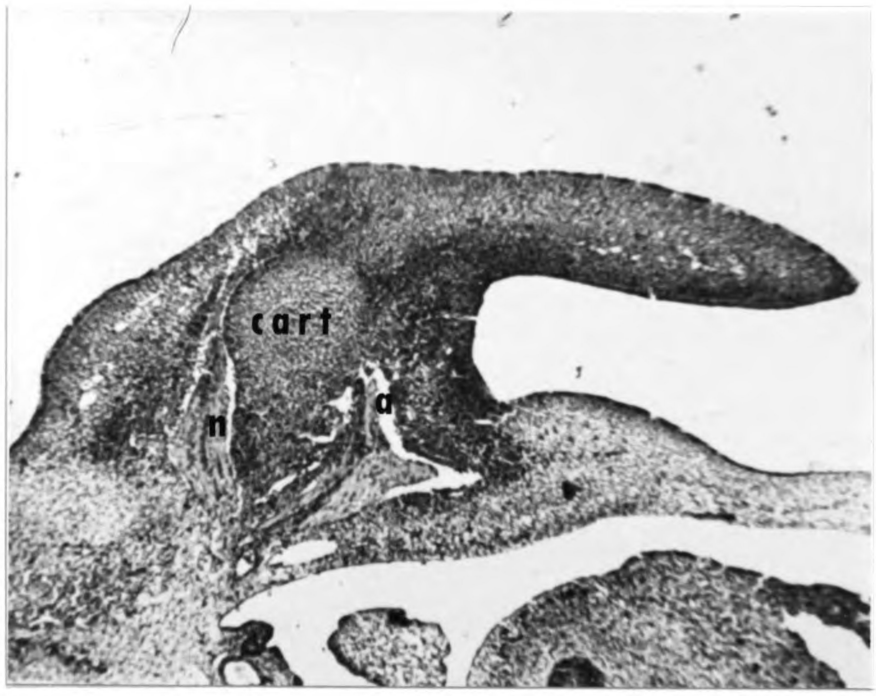
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Figure 5. Forelimb. Stage 31. A periosteal collar has formed around the shaft of the cartilaginous long bones. Note the scapula (sc), humerus (hu), radius (ra), and ulna (ul). H. & E. X40.

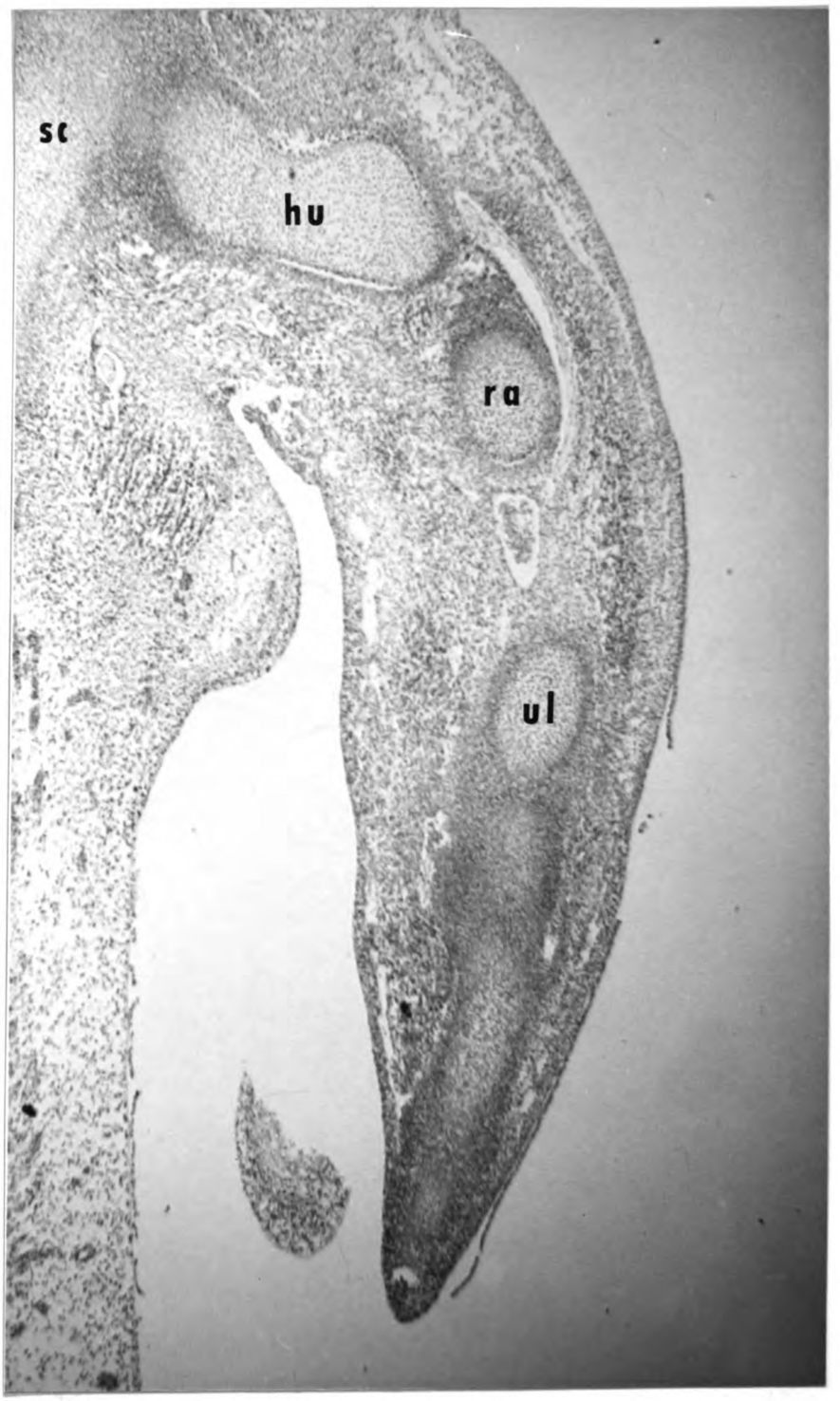
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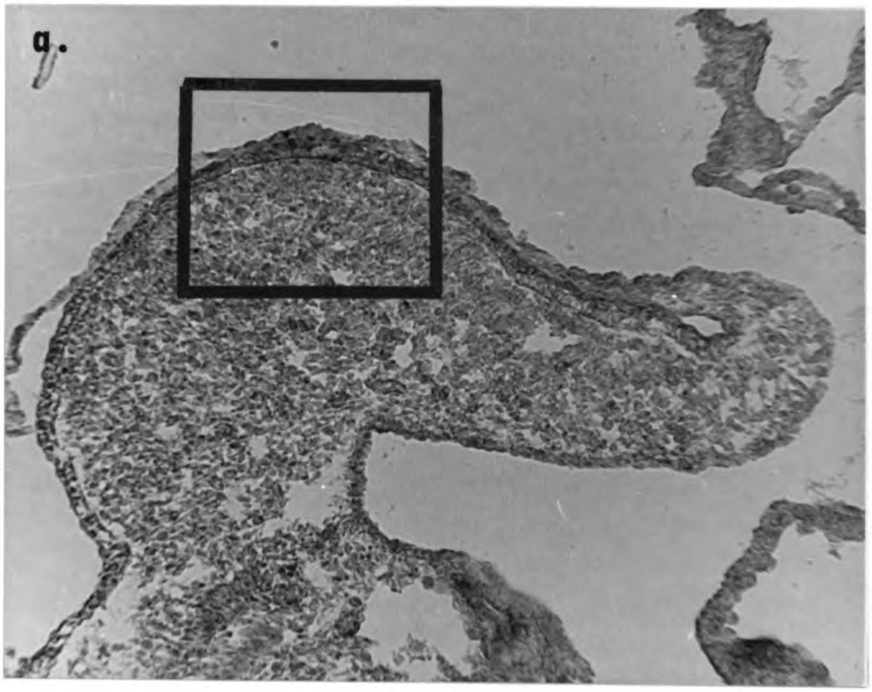
Figure 6. Forelimb bud. Stage 19. Activity is slight in the mesoderm (mes), ectoderm (ect), and amnionic sac (as). Azo dye technique for AcP.

a. X140.

b. X560.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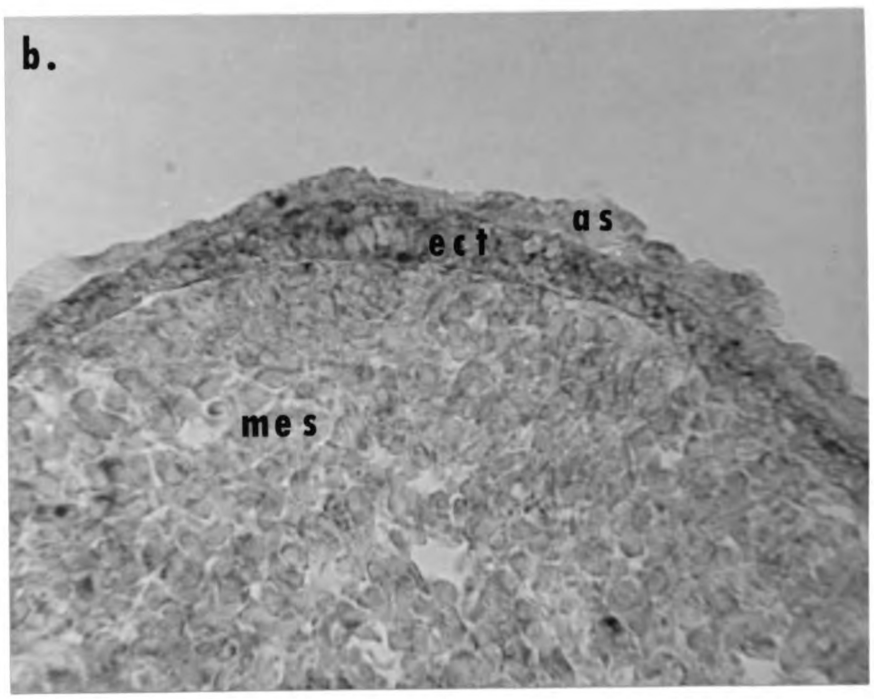
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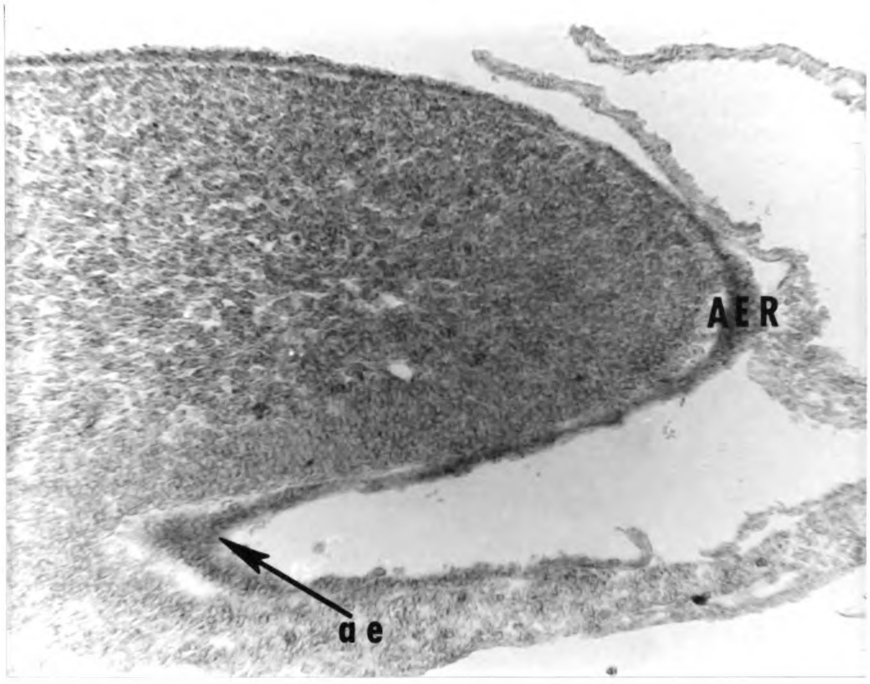
Figure 7. Forelimb bud. Stage 21. Note the moderate activity in the AER and in the axillary ectoderm (ae). Azo dye technique for AcP. X140.

Figure 8. AER. Stage 21. Cells of the ridge radiate out from a central core as identified by the arrow. Azo dye technique for AcP. X350.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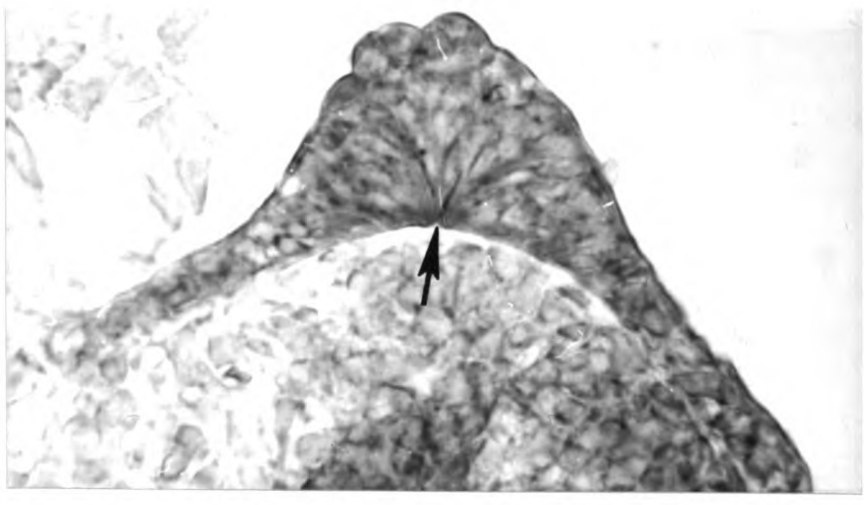


Figure 9. AER. Stage 21. Note the intense reaction in the core of the AER. Azo dye technique for AcP. X700.

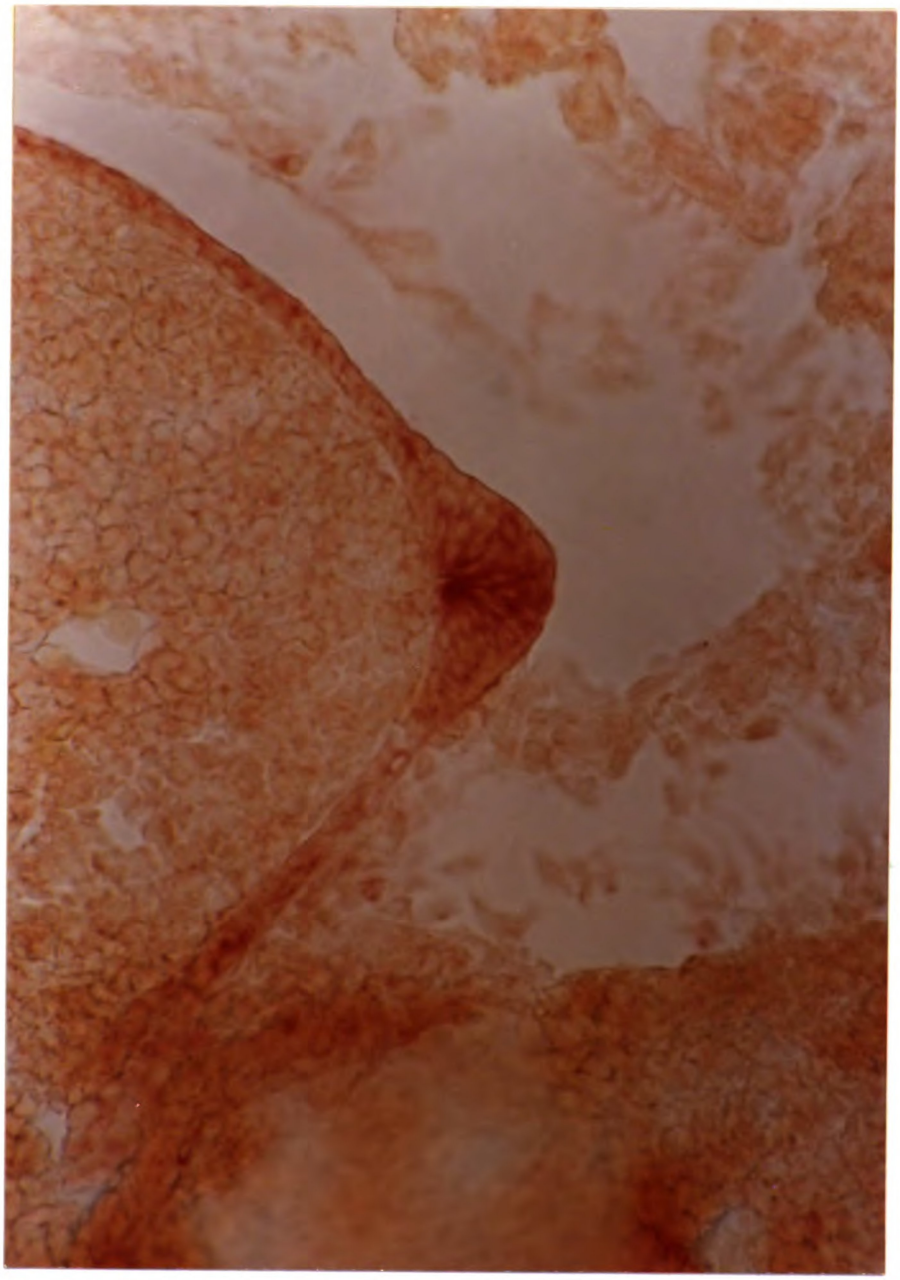
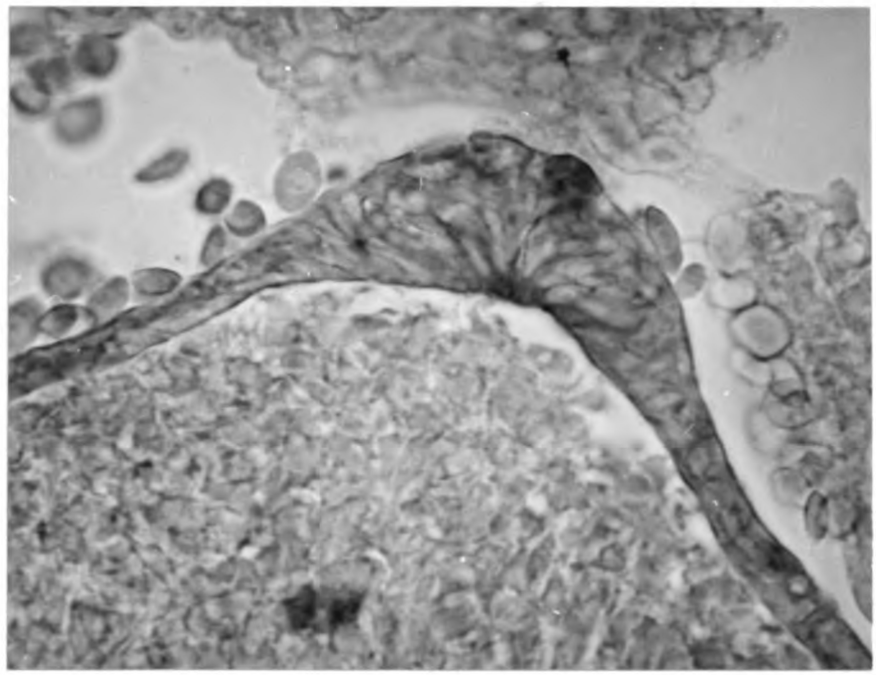


Figure 10. AER. Stage 24. Two centers of activity have developed, one in the central core and one in the periphery. Azo dye technique for AcP. X360.

Figure 11. Forelimb bud. Stage 24. Activity in the axillary ectoderm (ae) has decreased considerably. Azo dye technique for AcP. X140.



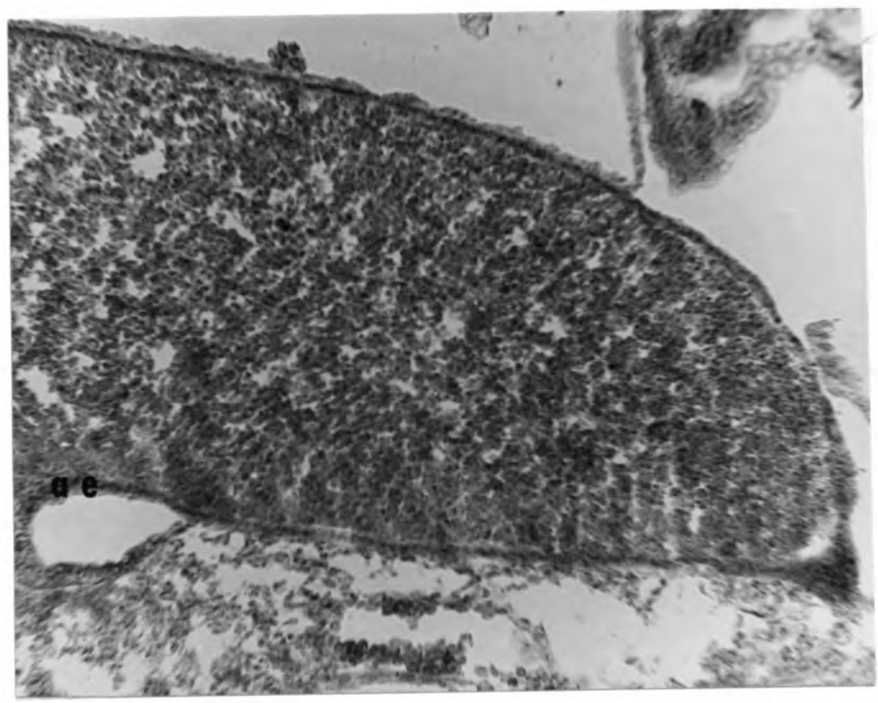

a e

Figure 12. Proximal portion of forelimb. Stage 27. The mesoderm, which is condensing to form cartilage, shows an almost total lack of activity. The ectoderm around the limb shows an intense activity in almost all cells. Azo dye technique for AcP. X110.

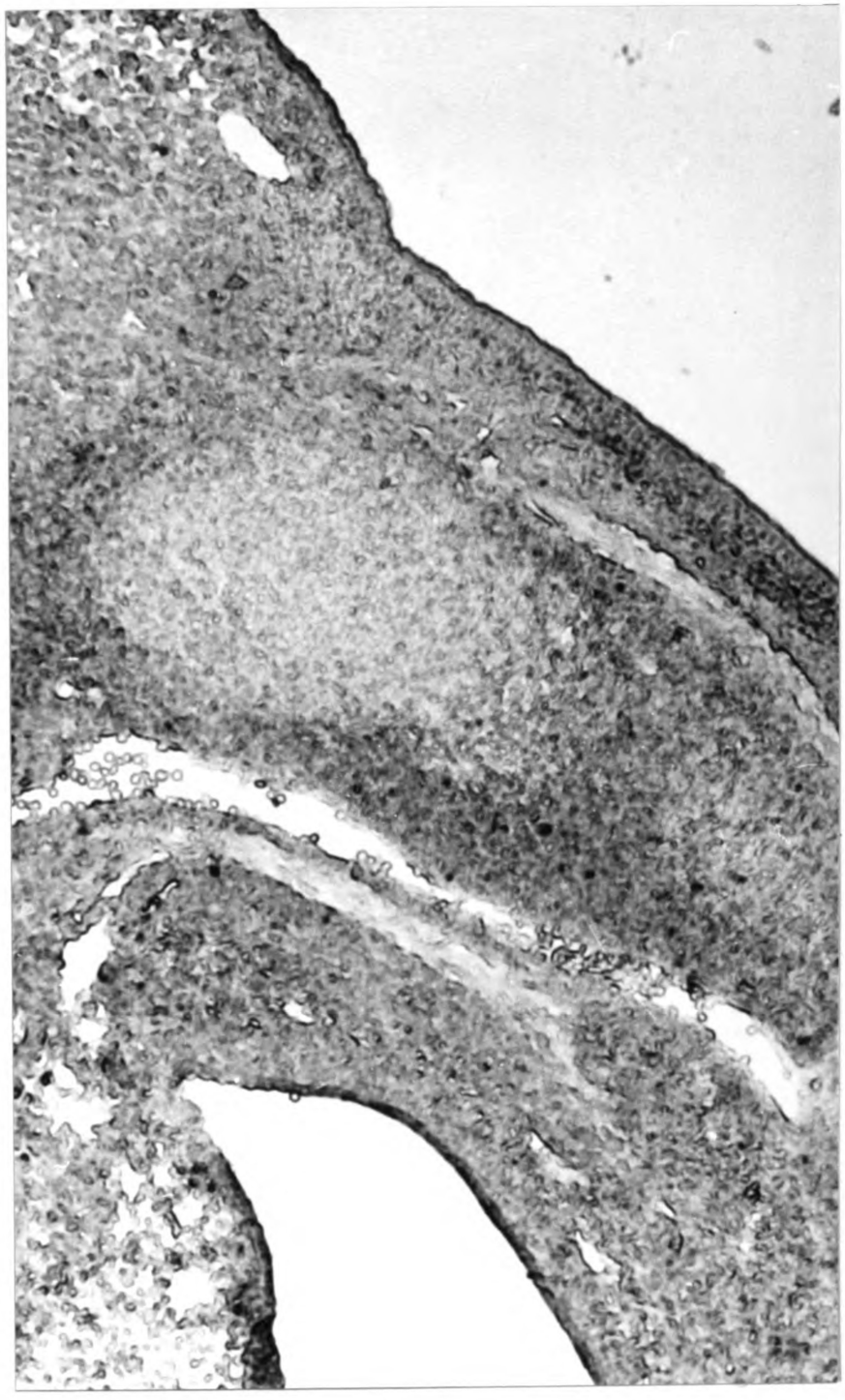
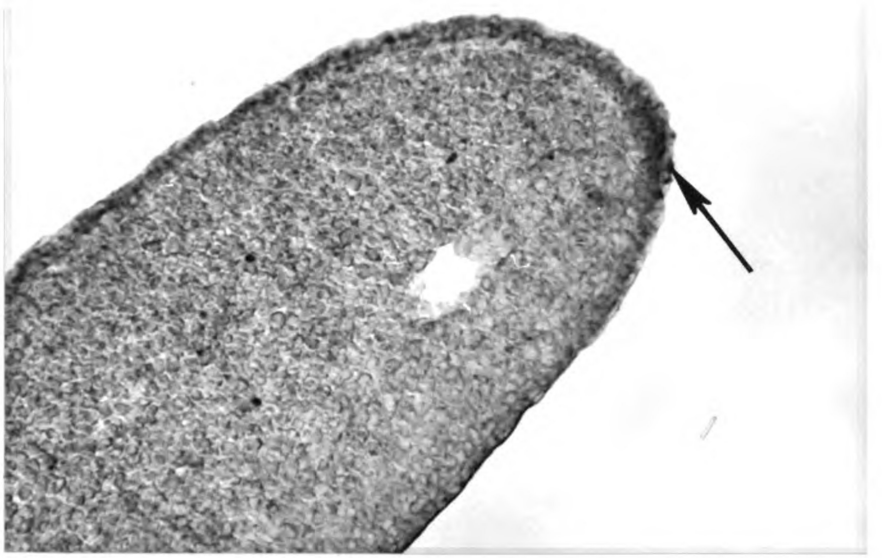
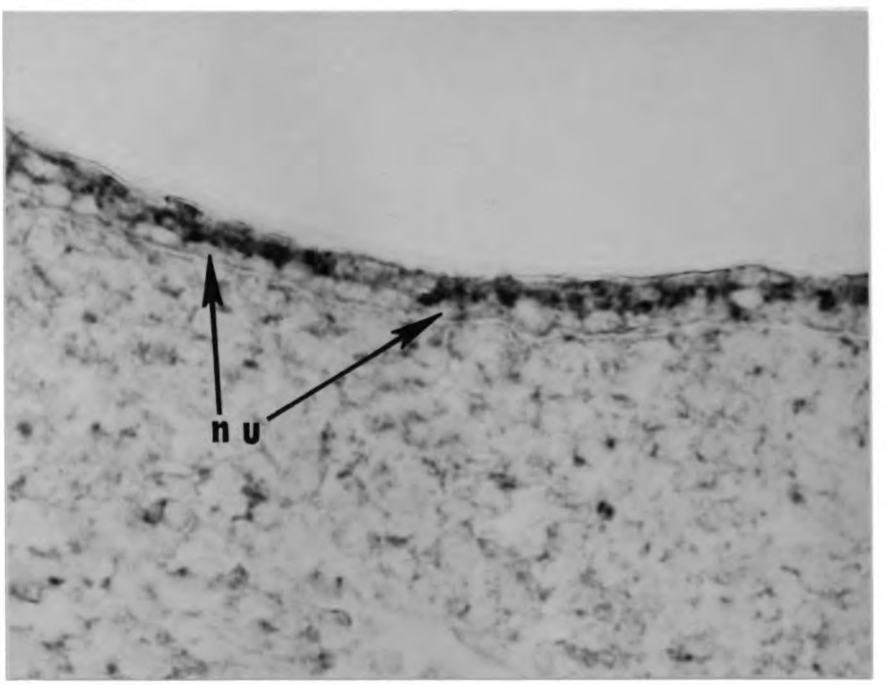


Figure 13. Distal tip of forelimb. Stage 27. An intense activity remains in the ectoderm which once represented the AER, as indicated by the arrow. The size of the ridge is now markedly reduced to only one or two layers thick. Azo dye technique for AcP. X225.

Figure 14. Skin of the forelimb. Stage 27. Activity appears to be more concentrated along the apical portion of the cells due primarily to the presence of the nucleus (nu) along the proximal border. Azo dye technique for AcP. X710.




nu

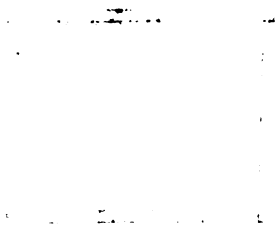
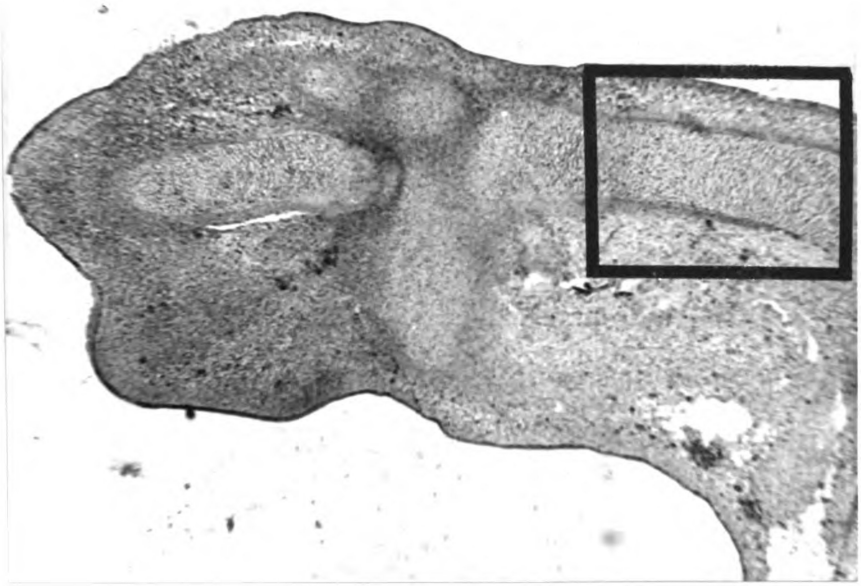


Figure 15. Forelimb. Stage 31. Azo dye technique for AcP.

a. Moderate activity is observed in the ectoderm around the forelimb. Slight activity is seen in the undifferentiated mesoderm. X24.

b. The cartilage (cart) exhibits moderate activity as does the periosteal collar (pc) around it. X140.

a.



b.

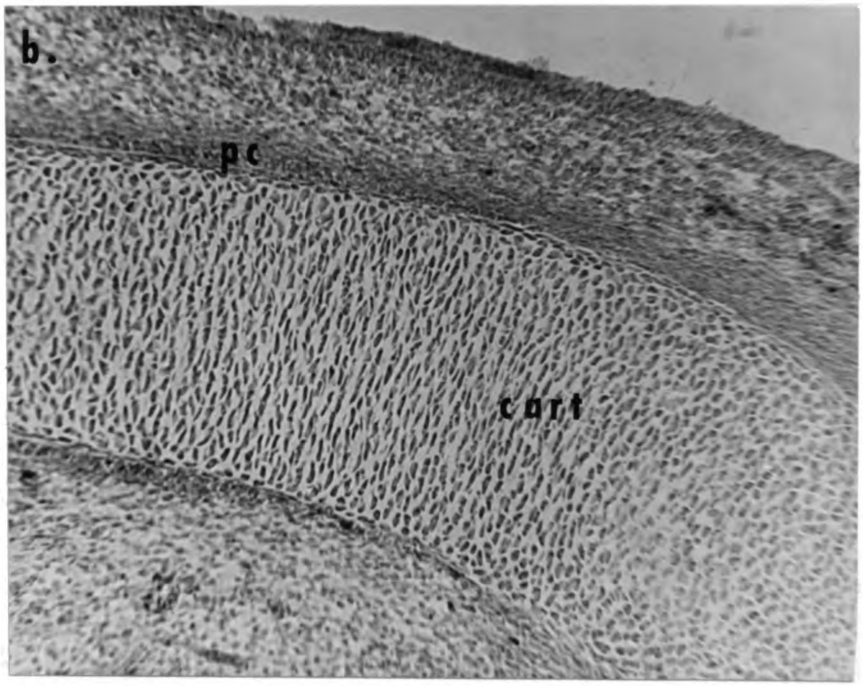
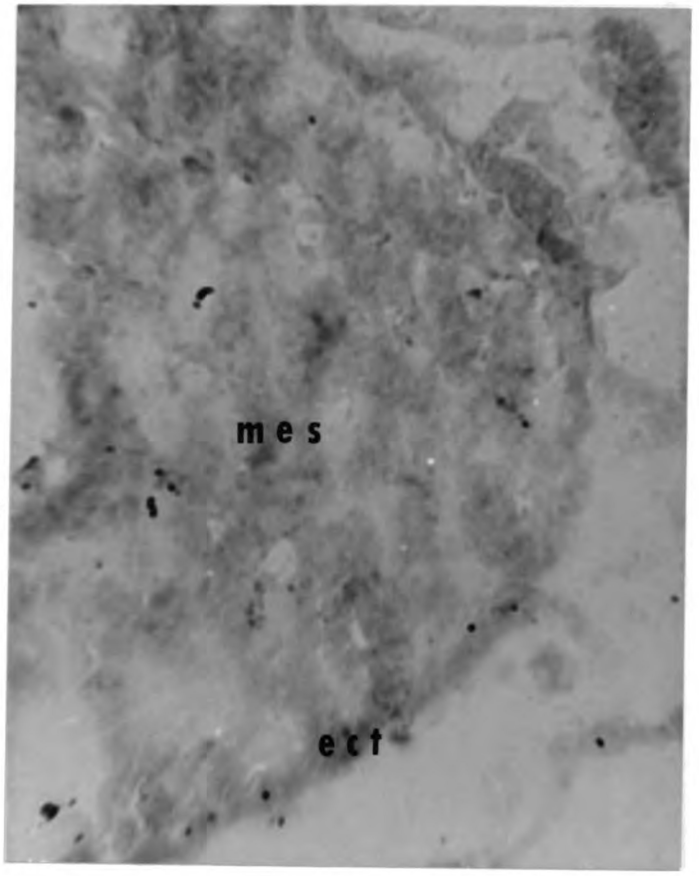


Figure 16. Forelimb bud. Stage 19. Slight activity is evenly distributed throughout the ectoderm (ect) and mesoderm (mes). Lead nitrate method for ATPase. X225.

Figure 17. Forelimb bud. Stage 21. The ventral mesoderm (vm) exhibits moderate activity. Lead nitrate method for ATPase. X56.


mes
ect

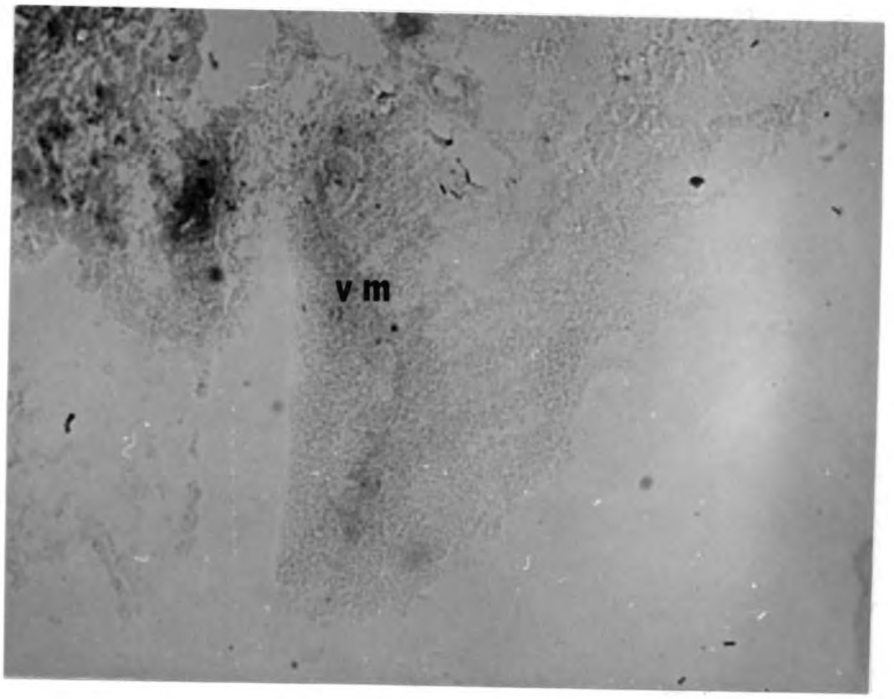

vm

Figure 18. Forelimb bud. Stage 24. The ventral mesoderm (vm) shows moderate activity. The AER has become moderately active. Lead nitrate method for ATPase. X112.

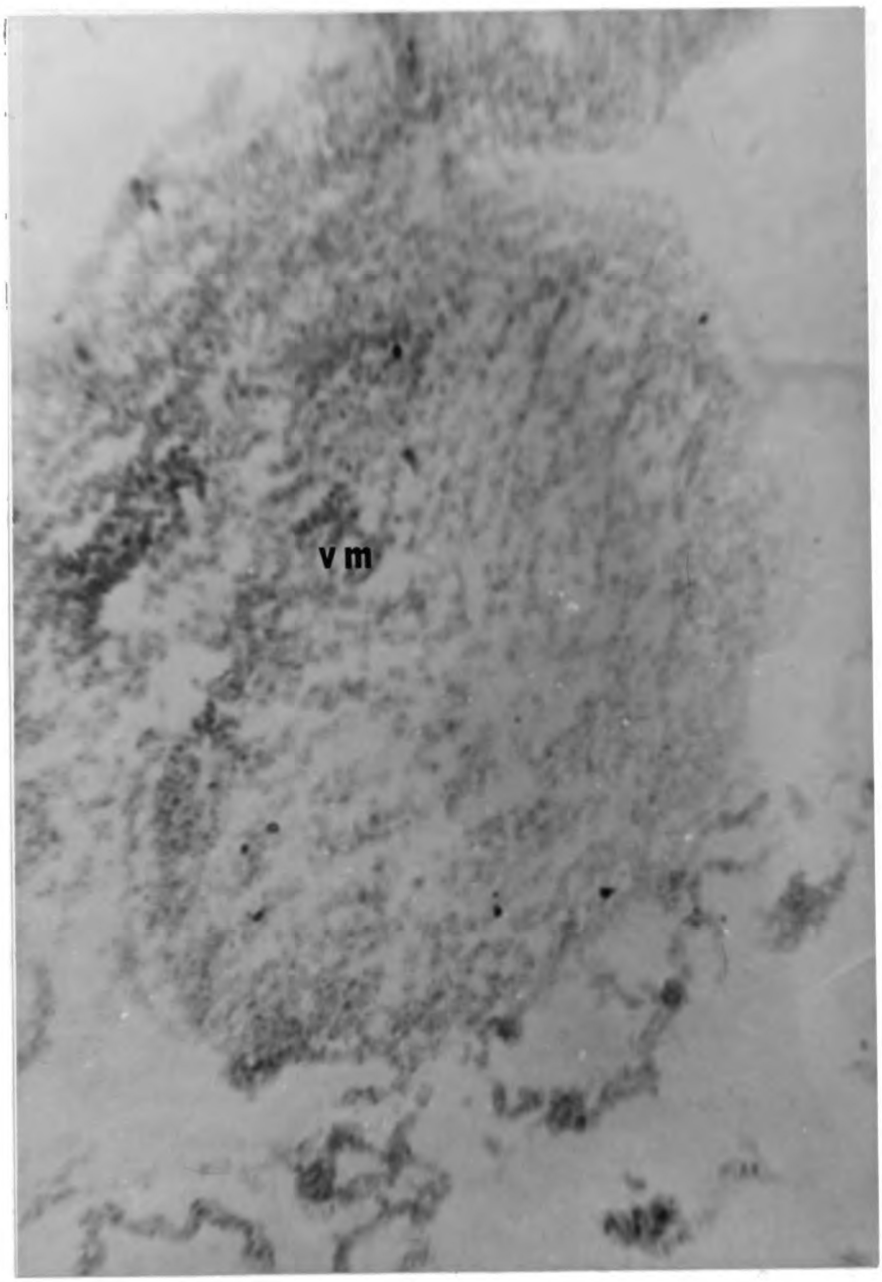
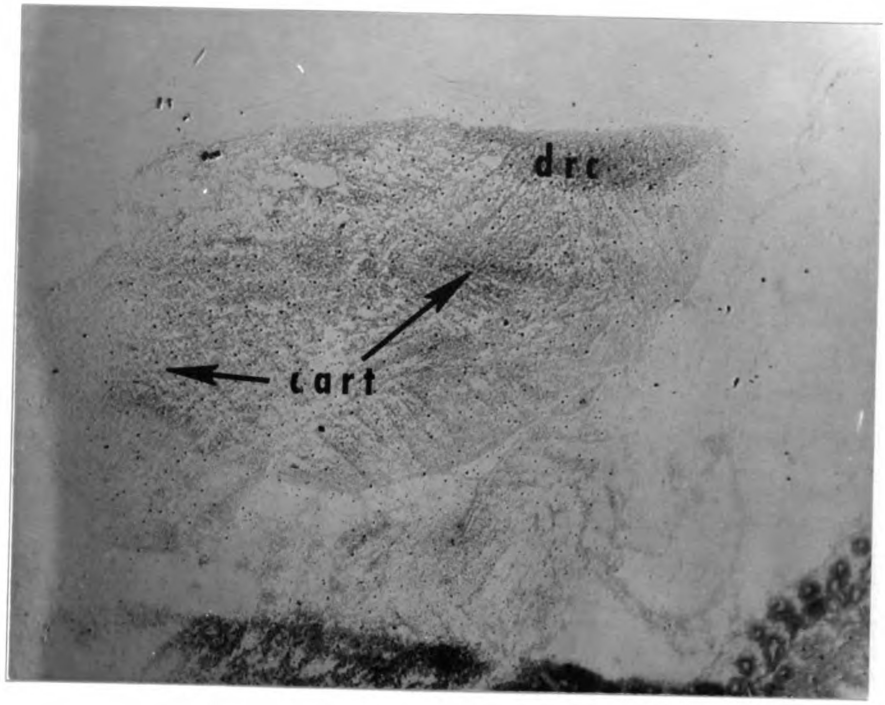

v m

Figure 19. Forelimb. Stage 27. Developing cartilage (cart) and a distal reactive center (drc) exhibit moderate activity. Note the AER has now disappeared. Lead nitrate method for ATPase. X36.

Figure 20. Developing bone in cross section. Stage 31. Note the intense reaction in the cartilage (cart) and in the periosteal collar (pc). Lead nitrate method for ATPase. X140.


drc
cart

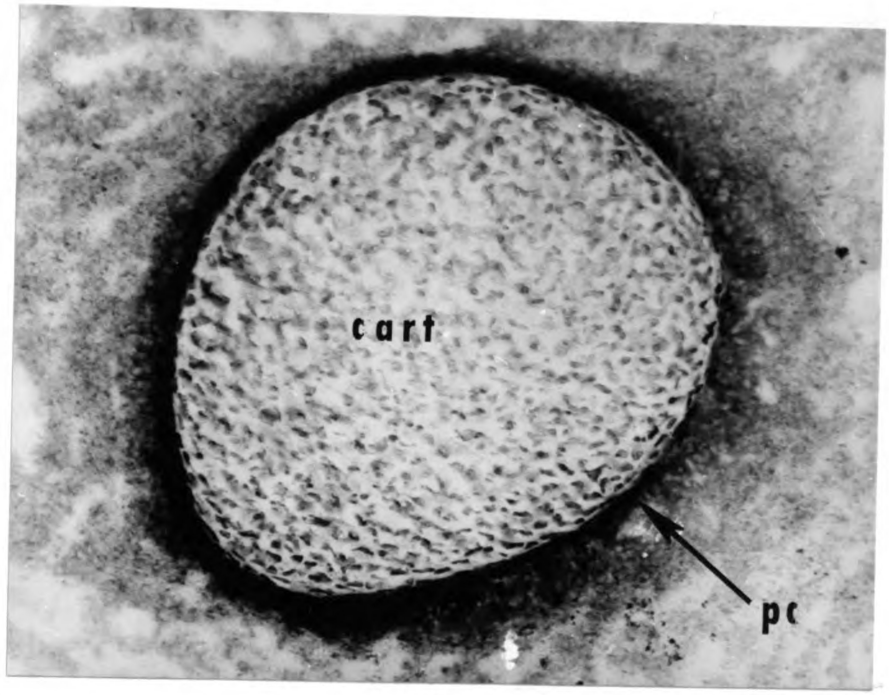

cart
pc

Figure 21. Developing bone. Stage 31. Intense periosteal collar activity appears only in the midshaft region of the diaphysis. Lead nitrate method for ATPase. X112.

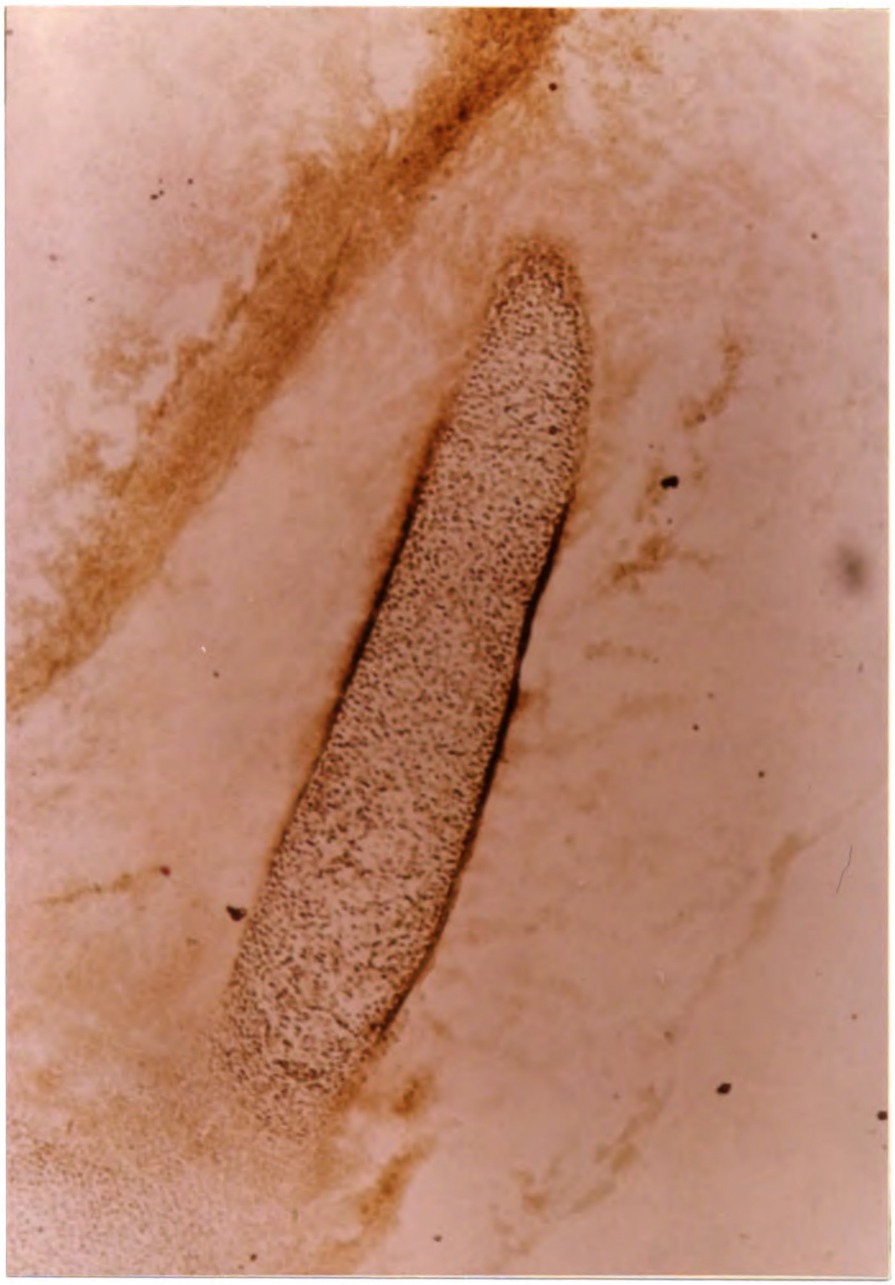
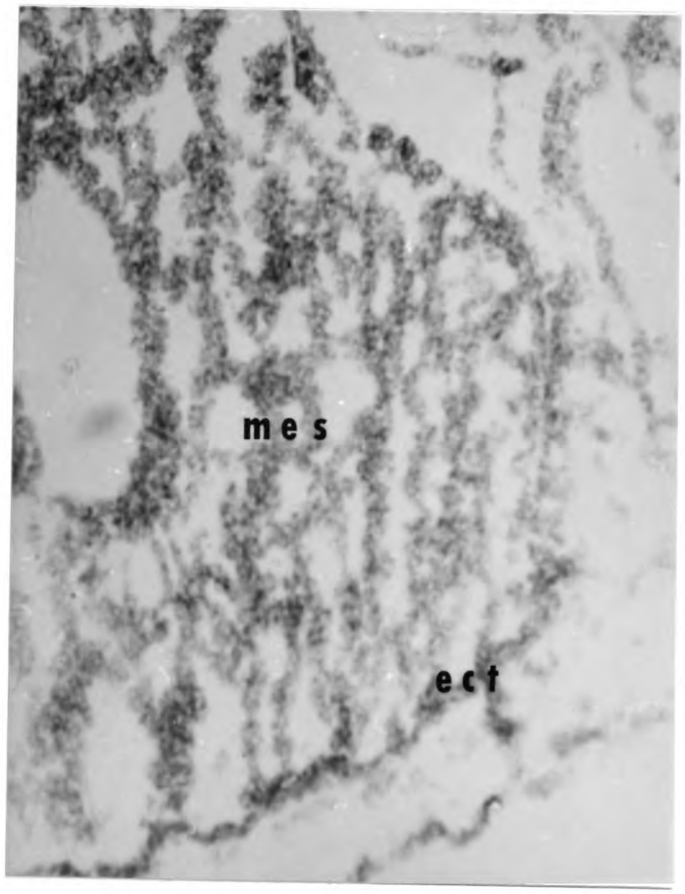


Figure 22. Forelimb bud. Stage 19. Note the slight activity evenly distributed throughout the mesoderm (mes) and ectoderm (ect) of the bud. Nitro-BT method for LDH. X225.

Figure 23. Forelimb bud. Stage 21. The AER now gives a moderate reaction (arrow). Moderate activity is also observed in the ventral mesodermal condensation (vmc). Nitro-BT method for LDH. X56.


mes
ect

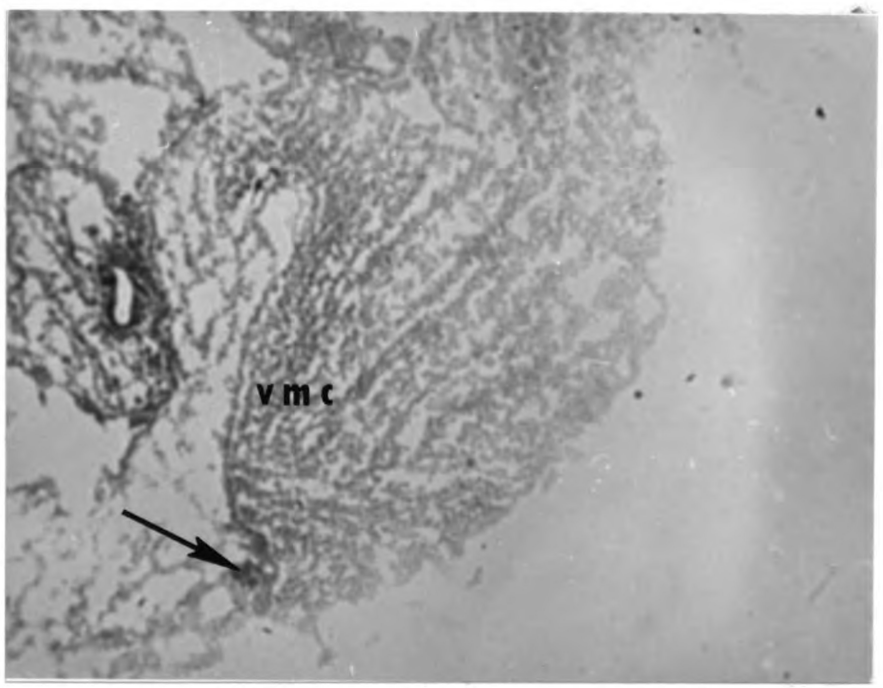
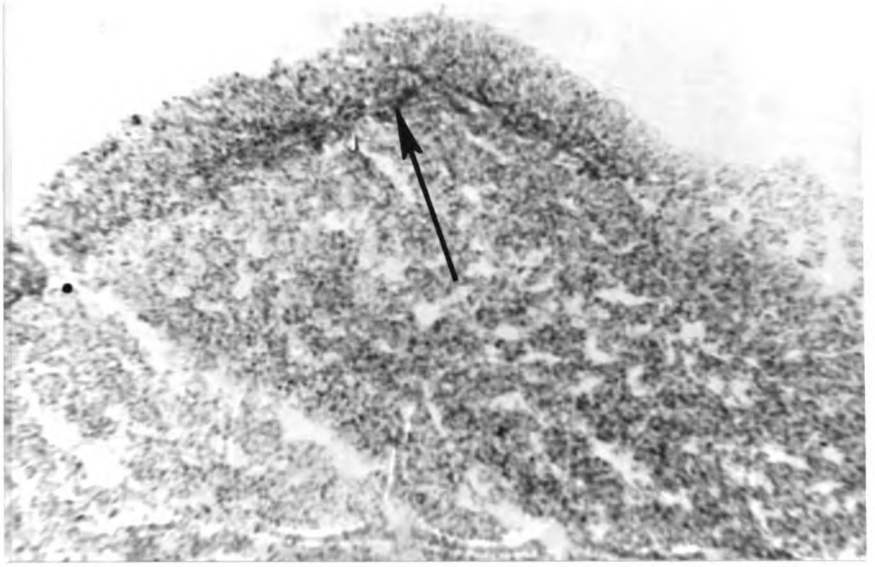

vmc

Figure 24. AER. Stage 24. Moderate activity is observed in the AER. The mesodermal cells just under the AER exhibit an intense reaction (arrow). Nitro-BT method for LDH. X360.

Figure 25. Axillary region. Stage 24. Intense enzyme activity is seen in the ventral mesodermal cells just under the ventral proximal ectoderm (arrow). The activity extends into the body cavity. Nitro-BT method for LDH. X360.



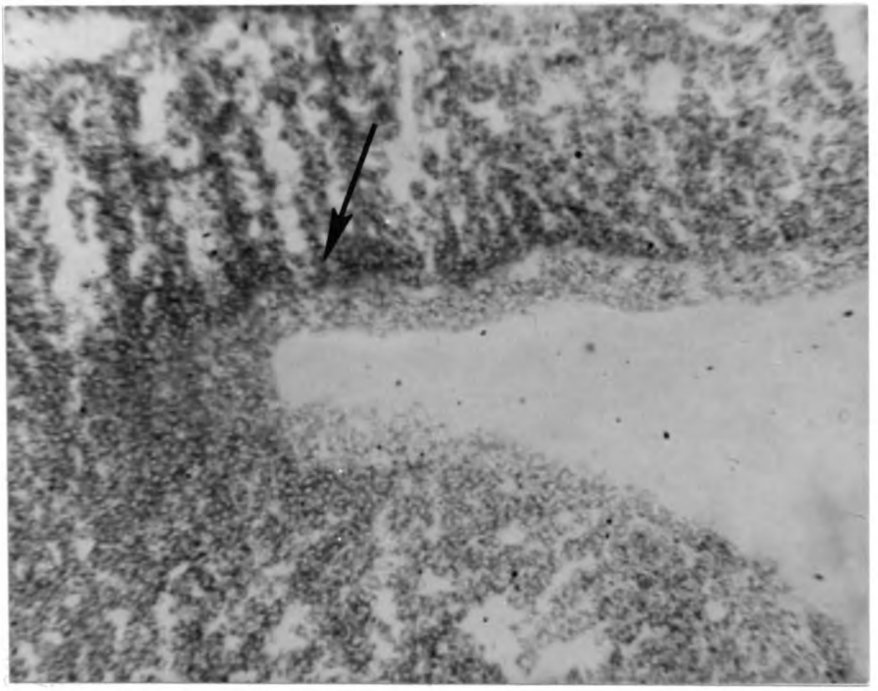


Figure 26. Forelimb. Stage 27. Two distinct centers of mesodermal activity exist. The ventral mesodermal condensation exhibits an intense reaction in the proximal portion just under the ventral ectoderm (1) and extends into the ventral body wall (vbw). Another site of moderate activity is in the dorsal proximal mesoderm (2), which extends into the dorsal body wall (dbw). Nitro-BT method for LDH. X112.

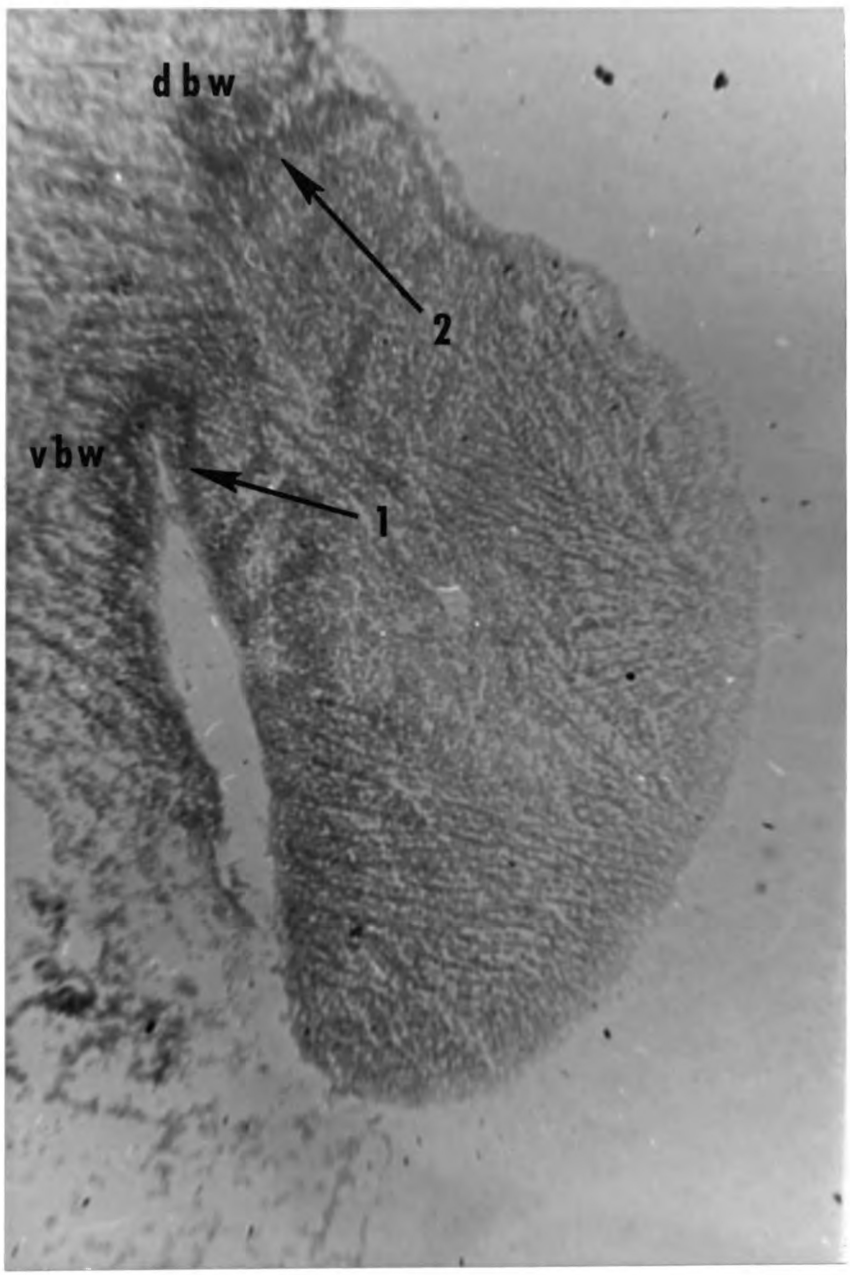

dbw
2
vbw
1

Figure 27. Axillary region. Stage 27. Note the intense activity in the row of mesodermal cells bordering the ventral ectoderm of the limb and extending into the ventral body wall (arrows). Nitro-BT method for LDH. X300.

Figure 28. Bone formation in cross section. Stage 31. The cartilage (cart) exhibits an intense reaction and the mesoderm (mes) gives only a moderate reaction. Nitro-BT method for LDH. X150.

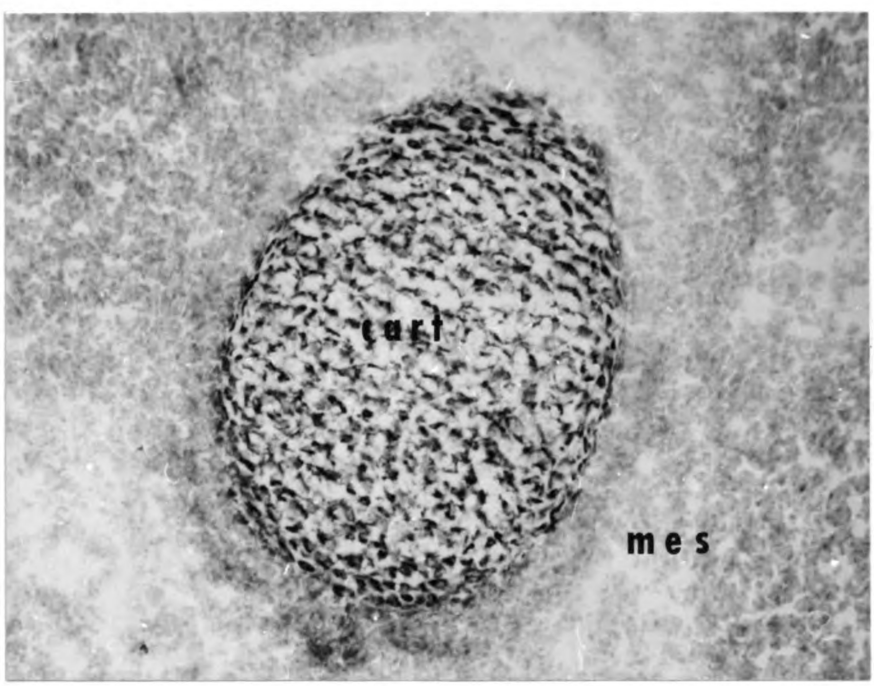
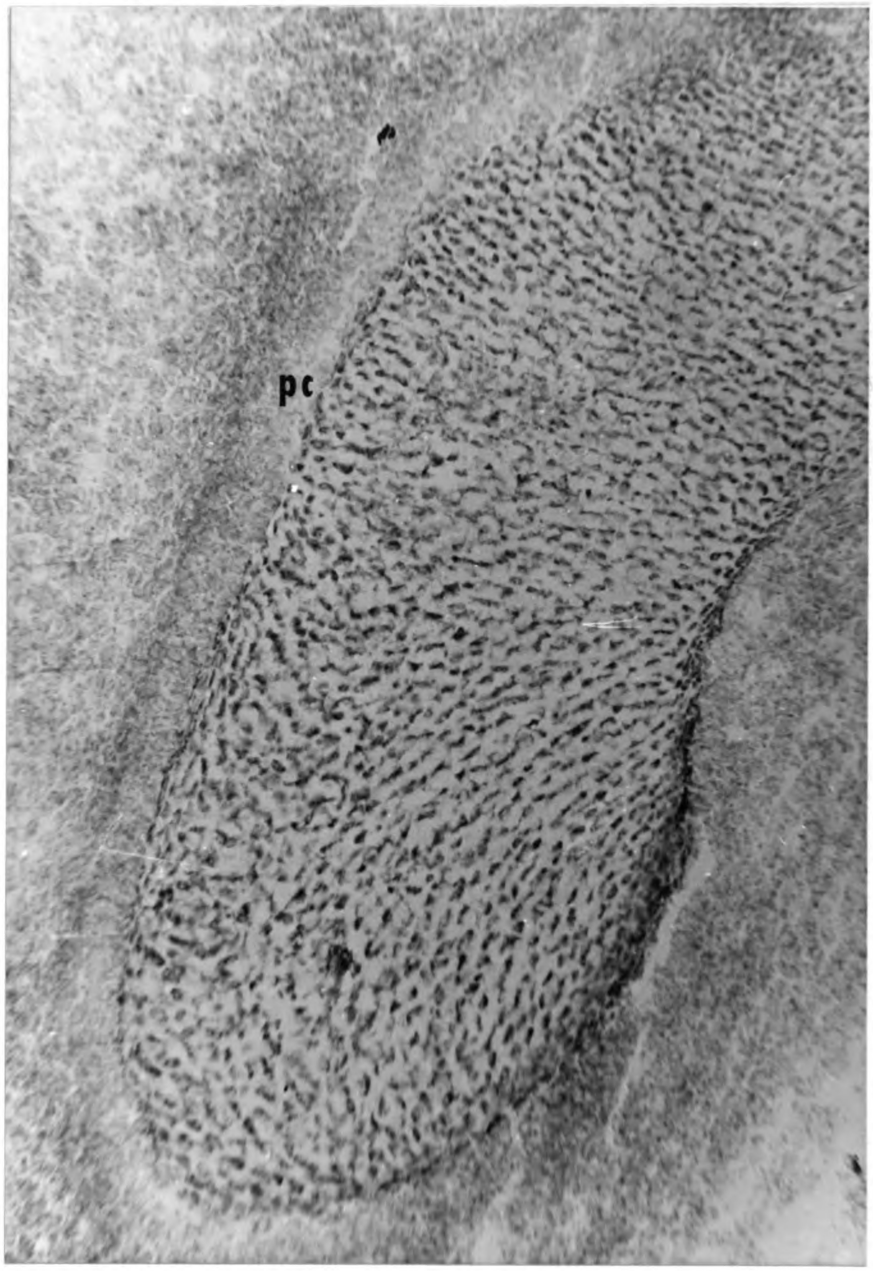

cart
mes

Figure 29. Developing long bone. Stage 31. The periosteal collar (pc) exhibits only a slight reaction. Nitro-BT method for LDH. X280.


pc