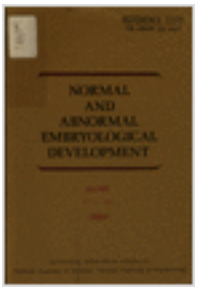

Normal and Abnormal Embryological Development: Proceedings of a Symposium (1967)

Pages
92

Size
5 x 9

ISBN
030934784X

Charles H. Frantz, Editor; Subcommittee on Child Prosthetics Problems; Committee on Prosthetics Research and Development; Division of Engineering; National Research Council

NORMAL AND ABNORMAL EMBRYOLOGICAL DEVELOPMENT

PROCEEDINGS OF A SYMPOSIUM
Held at
Chicago, Illinois

January 28, 1966

SUBCOMMITTEE ON CHILD PROSTHETICS PROBLEMS
COMMITTEE ON PROSTHETICS RESEARCH AND DEVELOPMENT
DIVISION OF ENGINEERING
OF THE
NATIONAL RESEARCH COUNCIL

Edited by
CHARLES H. FRANTZ, M.D.

Publication 1497
NATIONAL RESEARCH COUNCIL
NATIONAL ACADEMY OF SCIENCES NATIONAL ACADEMY OF ENGINEERING
Washington, D.C. 1967

Frontispiece: Human Embryo at the Sixth Week. (After Blechschmidt, E., The Stages of Human Development Before Birth.)

Available from
Committee on Prosthetics Research and Development
National Academy of Sciences
2101 Constitution Avenue
Washington, D.C. 20418

Library of Congress Catalog Card Number 67-61863

Foreword

Typically, the annual meeting of the Child Amputee Clinic Chiefs, sponsored by the Subcommittee on Child Prosthetics Problems of the Committee on Prosthetics Research and Development, National Academy of Sciences - National Research Council, is primarily concerned with surgical and prosthetic management problems. A striking addition to the program of the 1966 meeting involved presentation of the papers on normal and abnormal embryological development that are printed in this volume. In addition to the published material, Dr. Edgar Zwilling also presented a paper, "Abnormal Limb Development," which unfortunately was not available for inclusion in this report.

Organization of the symposium and publication of this report were part of the work conducted under a grant between the Area Child Amputee Center (Division of Services to Crippled Children, Michigan Department of Public Health) and the Children's Bureau, Department of Health, Education, and Welfare, and under a grant

between the National Academy of Sciences - National Research Council and the Children's Bureau, Department of Health, Education, and Welfare.

The assistance provided by the Association for the Aid of Crippled Children, through Chester A. Swinyard, M.D., to support the travel of Jean Milaire, Ph.D., is gratefully acknowledged, as are the contributions of Hector W. Kay, Shirley Furgerson, and Enid N. Partin in the preparation of this publication.

SUBCOMMITTEE ON CHILD PROSTHETICS PROBLEMS

COMMITTEE ON PROSTHETICS RESEARCH AND DEVELOPMENT

DIVISION OF ENGINEERING, NATIONAL RESEARCH COUNCIL

NATIONAL ACADEMY OF SCIENCES

NATIONAL ACADEMY OF ENGINEERING

Introduction

CHARLES H. FRANTZ, M.D.

We meet this morning to pursue a common interest. Our clinical endeavors are focused on child amputees and on children with limb malformations. Utilizing advanced prosthetic techniques, our goal is the restoration of limb function, in the hope that these handicapped children will be afforded the opportunity of assuming some degree of economic independence in the future.

We are all aware that the patterns of these limb malformations are set during the embryonic period of pregnancy. Whether they are the result of variations within the uterine environment or are genetically coded does not change our clinical problems.

We hope that in the future ways and means may be found to modify or alleviate some of the common abnormalities. To arrive at a better understanding of the mechanisms at work, we must turn to our contemporaries in research.

Most of us have been far afield from laboratories for many years. Much has happened in the interim. The advances that have been

made in understanding cell structure, composition, and chemistry are deeply significant. Although we clinicians tend to focus our attention on structural defects, it is highly important that we also appreciate the biochemical aspects, the metabolic processes, and the variations that may prove to have teratogenic implications.

The concept of induction interactions between various tissues, wherein messages flash between germ layers, has been developed since we left our medical schools. The influences of DNA, RNA, and the mucopolysaccharides are beyond the scope of our clinical understanding, although we may, in a general manner, appreciate their significance.

Today we are fortunate in having an opportunity to listen to addresses by distinguished investigators in their respective fields of research. I am certain that the panel's discussion and correlation of facts and concepts relative to embryonic development will enlighten us and further stimulate our efforts within the parameters of clinical practice.

Normal Development of the Human Embryo

RONAN O'RAHILLY, M.D.
Chairman, Department of Anatomy
Saint Louis University School of Medicine
St. Louis, Missouri

"Pour connaître l'homme malade, il faut connaître l'homme sain." The importance of an appreciation of normal structure and function as a prerequisite to an understanding of the abnormal is evident all the way from gross appearances to ultrastructure and molecular biology, and from senility back to the zygote.

The curious myth, however, that human anatomy, whether adult or developmental, has been completely elucidated during the four centuries following Vesalius, needs, it seems, to be re-exploded at regularly recurring intervals.

The development of the heart, about which volumes have been written, may serve as an example. The point has been well made by a cardiologist, in discussing the reason for the current lack of interest in research in human embryology (Grant, 1962). ". . . [it] cannot be that all cardiac development problems are solved, for there is scarcely a single form of congenital heart disease the developmental mechanism of which is confidently known, and many

aspects of normal heart development are still obscure. . . . That cardiologists have not taken part in embryologic research might be of only passing interest were not classical embryologists turning away from organ differentiation in their research toward much earlier stages, toward cellular and molecular embryology."

The present remarks, which may serve to introduce the subject of human prenatal development, will be grouped under three headings: (1) a bird's-eye view of intrauterine existence; (2) the limb as an index of human development; and (3) the limb as an object of teratological study.

1. A SURVEY OF INTRAUTERINE EXISTENCE (Figure 1)

Intrauterine Life

Apart from a preliminary voyage of half a week along the uterine tube, the remaining 38 weeks of prenatal life are spent in an intrauterine environment. Intrauterine life, therefore, is practically synonymous with prenatal life.

Postovulatory Weeks

Pregnancy extends from fertilization to the onset of parturition, and prenatal age is the length of time after fertilization. Because ovulation, coitus, and fertilization are believed to be separated by at most one or two days, these three events are more or less interchangeable in expressing prenatal age. The uncertainty, however, of stating "five months," for example, should be apparent. Is the reference to lunar months or to calendar months? Is the measurement from the last menstrual period or from fertilization? The most satisfactory units of prenatal measurement, in the writer's opinion, are "postovulatory weeks," that is, the length of time after ovulation. Where the date of ovulation is not known, as is commonly the case, the number of postovulatory weeks has to be estimated from the developmental status and/or the size achieved by the embryo or fetus.

The Embryo

During the first postovulatory week of human development, the zygote undergoes cleavage to become a morula, which, on the

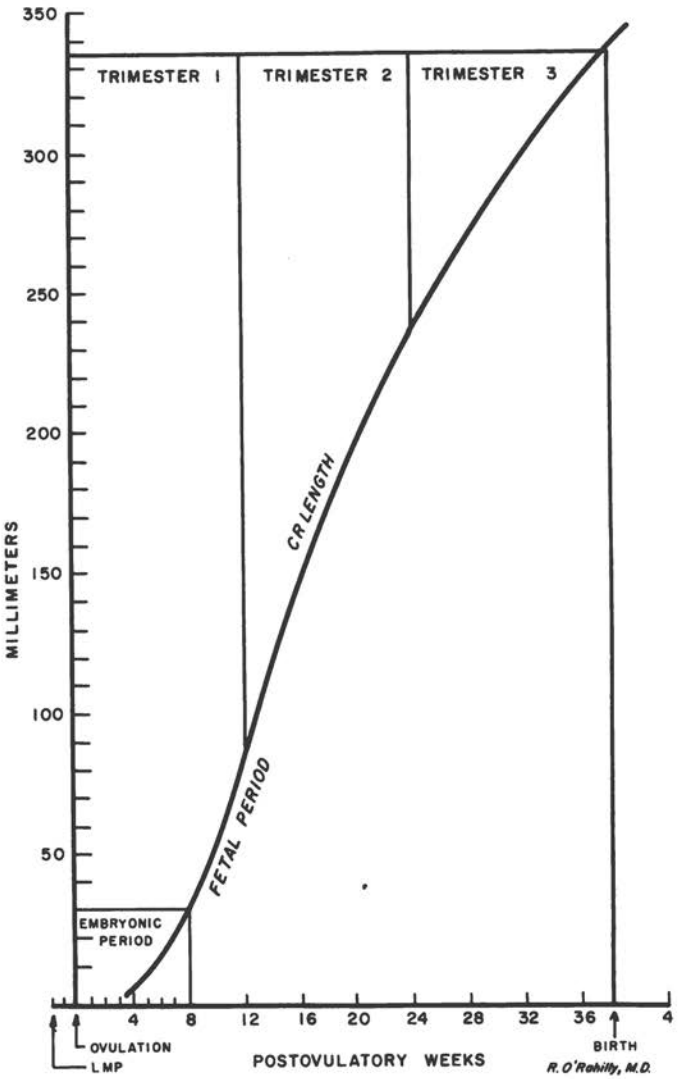

FIGURE 1 Intrauterine life. Graph of CR length, or sitting height, throughout intrauterine life. The ordinate indicates the CR length in millimeters while the abscissa represents the approximate age in weeks since the last ovulation. The CR line is intended to show the central trend, but considerable variation occurs. Prenatal life may be divided conveniently into the embryonic period proper, a particularly vulnerable interval occupying the first 8 weeks, and the fetal period. Division into three equal parts, or trimesters, is also shown. CR, crown-rump. LMP, last menstrual period.

appearance of a cavity within the mass of cells, is transformed into a blastocyst. As soon as the zygote undergoes cleavage, it is frequently termed an embryo. However, because the zygote gives rise to extraembryonic membranes as well, some authors use the term "embryo" only when the embryonic disc has formed (at about nine days). In general, however, the term "embryonic period" is employed from the commencement of prenatal life.

The Last Menstrual Period

The so-called menstrual age, that is, the length of time following the last menstrual period (LMP), was formerly supposed to provide an index of prenatal age, owing to the assumption that ovulation takes place precisely two weeks after the LMP. This assumption is unjustified, and delayed ovulation is now believed to account for instances of alleged prolongation of gestation.

Embryonic and Fetal Periods

Prenatal life may conveniently be divided into two eras: (1) the embryonic period proper, and (2) the fetal period.

The embryonic period proper consists of the first 8 postovulatory weeks, and during this interval the organism undergoes intense differentiation, whereby the vast majority of the body's organs are laid down. Already, for example, by 6 weeks the bronchi of the 10 bronchopulmonary segments can be clearly identified. The embryonic period proper has been divided into 23 stages, each of which is characterized by a number of external and internal morphological features, such as the external form of the limb buds, or the internal appearances of the developing eye. Development involves three interrelated although dissociable components: (1) increase in age; (2) increase in size, or growth; and (3) increase in maturity. The 23 embryonic stages are levels of maturity and help to liberate embryology in large measure from the variability of timing and of linear measurements. Incidentally, the term "stage" should no longer be recorded unless a system of staging, as distinct from mere measuring of the embryo, has been employed; for example, such an expression as "the 20-mm stage" should not be used.

At the end of 8 postovulatory weeks, the fetal period begins, and its onset is heralded by the first formation of marrow within the humerus. The emphasis in development now shifts from differentiation to growth.

Crown - Rump Length

The most generally useful measurement, particularly in the fetal period, is the crown - rump (CR) or vertex - breech length, devised by Arnold in 1887. This is the measurement in a straight line from the crown, or highest point, of the head to the lowest point of the breech. In young embryos the crown of the head lies immediately over the midbrain. The CR length should always be recorded in millimeters. It corresponds to the sitting height postnatally; it is used prenatally in preference to the standing height because of the flexion of the embryonic and fetal lower limbs.

Numerous tables, graphs, and formulas have been devised to convert the CR length into embryonic and fetal age. It must be admitted at the outset that considerable disaccord occurs among the estimates given by various authors. The following points may usefully be made. The embryonic period proper has been investigated extensively, and, although embryonic staging is the preferred method of indicating the developmental level attained, data are available to relate the CR length to age. At the end of this period, when the organism is 8 postovulatory weeks, the CR length is some 30 mm. The fetal period has not been investigated with similar care, and much greater variation is found. Halfway through prenatal life, that is, at 19 postovulatory weeks, the CR length is about 160 mm. However, in one study in which the mean halfway length was estimated to be 190 mm, it was calculated that four fifths of the halfway fetuses varied in CR length from 140 to 225 mm. Hence it seems clear that fetuses, like their postnatal successors, vary considerably in height for a given age.

In clinical usage, pregnancy is frequently divided into three equal periods, or trimesters. It is interesting to note that (1) at the end of the first trimester, the fetus is approximately a palm (ca 80 mm) in sitting height; (2) at the end of the second trimester it is approximately a span (ca 230 mm); whereas, (3) the sitting height does not reach a cubit (length of forearm and hand) until the infant has repeated its intrauterine age, that is, about nine months after birth.

2. THE LIMB AS AN INDEX OF DEVELOPMENT (Figure 2)

In the staging of embryos, whether of the chick or of the human, the appearance of the developing limbs is an obviously changing external morphological criterion.

By 4 postovulatory weeks, at approximately 5 mm CR length,

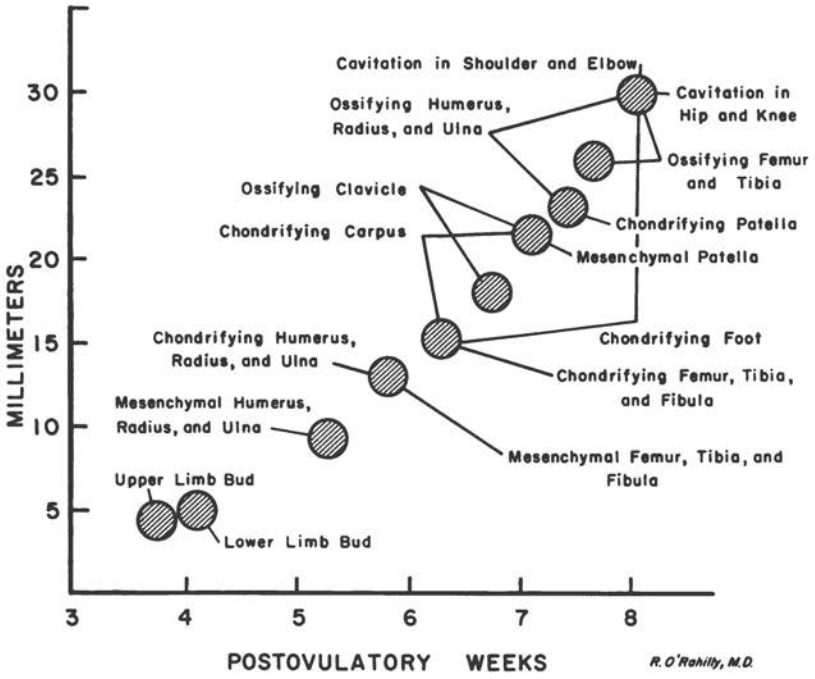

FIGURE 2 Early development of the limbs in the human embryo. The ordinate indicates the CR length in millimeters; the abscissa represents the approximate age in weeks since last ovulation. Each shaded circle represents a stage in development.

the human embryo displays a ring of thickened surface ectoderm extending from the ventral aspect of the head, along the body wall on each side, and ending on the ventral aspect of the caudal tip of the body. This ectodermal ring (Ektodermring) includes the nasal, lens, and otic discs (or placodes, as they are frequently called), and also the ectoderm of the pharyngeal arches. The limb buds arise dorsal to the ring, but their ventral aspects are covered by it.

Each limb bud appears as an elevation of the nonsegmented part of the body wall. The cells of the bud are derived from two sources, namely, the covering ectoderm and the mesoderm of the lateral plate. The causal interrelations between these two tissues have been the subject of extensive experimental work and much controversy. It is now believed that the limbs receive no contribution from the somites. The various segments of each limb are individuated in proximodistal sequence. The more proximal portion of the

hand, for example, is apparent before the fingers become defined. Finally, it may be pointed out that throughout prenatal life the development of the upper limbs is slightly more advanced than that of the lower limbs.

For detailed embryological analysis, it is essential to follow both the external and the internal development of the limbs in terms of precisely defined stages. However, to spare the reader who is not familiar with the numbering system of human embryological stages, the following summary has been translated into the more imprecise language of chronological age.

Four Postovulatory Weeks

The limb buds appear as slight elevations, the upper limb buds slightly before the lower. The width, that is, the distance from the body wall to the apex, soon comes to equal the length, which is the rostrocaudal dimension measured along the body wall. Thereafter, the limb buds elongate rapidly. The ventral surface and the margin of the limb buds display an ectodermal thickening, which rapidly forms an ectodermal ridge. A marginal vessel is found subjacent to the ectodermal ridge.

Five Postovulatory Weeks

In the upper limb bud, a hand plate becomes demarcated. The scapula, humerus, radius, and ulna can be identified as mesenchymal condensations. These skeletal cell groups, or blastemata, are derived from the central mesoderm of the limb bud. A blood circulation has become established within the bud, and nerve trunks from the neural tube are growing into the base of the bud.

In the lower limb bud, the caudal half appears to taper, and, at the tip, a foot plate begins to develop. The ectodermal ridge extends along the preaxial border and around the tip of each limb bud, part way onto the postaxial border.

Six Postovulatory Weeks

The margin of the hand plate develops a crenated rim because of the appearance of finger rays. Chondrification commences in the humerus, radius, ulna, and metacarpals. The clavicle and most of

the hand are present as mesenchyme. Muscle groups and the major branches of the brachial plexus can be distinguished.

In the lower limb, toe rays appear. The ilium, femur, tibia, fibula, and foot ray are present as mesenchyme, but chondrification commences rapidly in the femur, tibia, and fibula.

Seven Postovulatory Weeks

The limb buds extend ventrally, but indications of the elbow and the knee are evident. The ectodermal ridges are no longer found. The limb buds have well-defined longitudinal axes, ventral and dorsal surfaces, and preaxial and postaxial borders. The term "preaxial" is used for the rostral border, that on which either the thumb or the big toe is situated. The "postaxial" is the opposite, or caudal, border. The adult location of the big toe medially and the thumb laterally depends on (1) an apparent medial rotation of the foot on adoption of a plantigrade progression, and (2) the definition of the anatomical position as an artificial but useful convention whereby the palms face forward, thereby maintaining the radius and the ulna in parallel.

Ossification begins in the clavicle, which is the first skeletal element to show this phenomenon. Chondrification commences in the carpus and in the proximal, middle, and distal phalanges.

In the lower limb, chondrification occurs in the ilium and in the foot. The patella appears as a mesenchymal condensation, and the site—"interzone"—of the hip and knee joints is evident.

Eight Postovulatory Weeks

At the end of the embryonic period proper, when the CR length is 30 mm, the external and internal features of the body are well developed. The skeletal elements of the limbs are present as cartilaginous models, the major joints are well advanced, and certain beginnings of bone formation are encountered. In brief, by this time the skeleton is, in general, a replica of the adult arrangement.

The upper limbs, which are slightly bent at the elbows, extend ventrally. They are rotated medially in such a way that the palms face toward the heart. Bony collars are appearing in the radius, ulna, and distal phalanges, and indications of bone formation may be found also in the scapula. Cavitation is commencing in the shoulder, elbow, and wrist.

The lower limbs, which are bent at the knees, also extend ventrally. The soles face each other, and the toes of one foot generally touch those of the other, resulting in an impression of what may be termed praying feet. The patella is undergoing chondrification, and bony collars are found in the femur and the tibia. Cavitation is under way in the hip and the knee.

The sequence of chondrification in the hand and foot is by no means similar to the more familiar sequence of subsequent ossification. In general, the various digital elements chondrify in proximodistal order: metacarpals or metatarsals, proximal, middle, and distal phalanges. With regard to the mediolateral arrangement, the various elements (e.g., metacarpals or metatarsals, proximal phalanges, middle phalanges) of digits 2 to 4 chondrify more or less simultaneously (3 and 4 probably slightly ahead of 2), and are followed by digit 5, and finally by digit 1. In the case of the distal phalanges, however, the order is 1, then 2 to 4, and finally 5. The above remarks, needless to add, refer to the human species.

An example will illustrate the differing sequences of chondrification and ossification. Although, in the case of the metacarpals and metatarsals, the sequence of chondrification and diaphysial ossification is generally the same (2 to 4 before 5, and 5 before 1), the phalanges begin to chondrify in proximodistal sequence, whereas in diaphysial ossification the order is distal, proximal, and middle.

Fetal Period

Clearly it is not practicable here even to attempt to summarize the many changes that occur in the limbs during the fetal period. In the case of the skeleton, for example, numerous bone collars appear and are followed by vascular invasion of the shafts, resulting in the formation of ossific centers. Indeed, as is well known, the origin of ossific centers is by no means confined to prenatal life, and many more centers are encountered during infancy and childhood.

3. THE LIMB AS AN OBJECT OF TERATOLOGICAL STUDY

It is conceivable that, under abnormal conditions, the development of a skeletal element, such as the tibia, might fail to take place, or, having commenced, might fail to proceed further. This is the concept of "developmental arrest," namely, that congenital defects

represent a failure to complete some normal developmental process. Although this idea may serve in some instances, its role has been overemphasized, and it is now realized that it is merely one mechanism in teratogenesis. Anomalous limbs in mice afflicted with congenital absence of the tibia were studied more than 30 years ago (Hovelacque and Noel, 1923). The anomaly was recognizable very early during embryonic development, that is, at the time the skeletal blastema appeared. A fibrous tract developed in place of the tibia, and, in some instances, cartilaginous nodules appeared at its upper end. The vascularization of the limb bud, however, was normal.

In these mice, there is clearly a point in embryonic development by which the malformation must appear if the anomaly is going to develop at all. Such a point is known as the teratogenetische Terminationspunkt of Schwalbe but, like the idea of developmental arrest, it has been considerably overworked in teratology. The dangers have been illustrated in an instructive series of experiments in which hens were fed with wheat containing selenium (Gruenwald, 1958). Many of the resultant embryonic abnormalities were found to be due to degenerative changes in previously well-formed parts. In other words, secondary destructive processes have to be taken into account as well as faulty primary formation. In considering the tibia, therefore, it must be recognized that a possibility exists that sometimes this skeletal element might develop and later undergo regressive changes.

After this brief discussion of deficiency, the rather different problem of duplication may next be considered. Recently, the early origin of polydactyly has been followed carefully in embryonic mice that, owing to the possession of a special gene, are subject to this anomaly (Forsthoefel, 1963). Extra blastemata for extra toes were found as mesenchymal condensations, that is, very early in development, and these extra digits then underwent the usual sequence of chondrification and ossification.

In the normal human embryo, the metatarsals have appeared first in mesenchyme and then in cartilage by 7 postovulatory weeks, and hence their number must have been determined before that time. Hence, if seven metatarsals were to develop instead of five, that number would have to have been determined prior to 7 weeks. This Terminationspunkt, or latest point in embryonic life at which the anomaly can develop, is clearly on much more secure ground than the case of the tibia, because subsequent regressive changes would not account for the presence of the extra toes.

Finally, and more curious still, is the combination of deficiency

and duplication sometimes found in the one anomalous limb. For example, in the special mice referred to above, the hindlimb was not infrequently characterized by both absence of the tibia and preaxial polydactyly. It has been proposed that such a seemingly paradoxical anomaly "may result from an excess outgrowth. . . which occurs relatively late, involves only the digital area, and attracts some of the tissue immediately proximal to the area of excess outgrowth. The key to whether proximal deficiencies will be encountered is the time of onset of excess outgrowth. If it starts early there will be a sufficient base for outgrowth and relatively little competition for proximal tissues; if excess outgrowth starts later, proximal tissues may be attracted to the region of outgrowth" (Zwilling and Ames, 1958).

That these anomalies in laboratory animals have their counterpart in the human will become clear from the following case report, which will serve as a conclusion to the present paper (Figure 3).

In Vancouver, British Columbia, an accident involving the collision of two automobiles occurred on 12 March. A woman in one of the cars was several weeks pregnant at the time. Some six months later a boy was born, but his right leg was deformed by a partial absence of the tibia (tibial hemimelia), and his right foot presented seven toes (preaxial polydactyly). A claim was entered to show that the deformity arose as a result of antepartum maternal trauma. Expert witnesses, among whom the writer was privileged to be included, were called to testify, and they gave evidence on the defendant's behalf.

The accident took place 12 weeks after the LMP. If the assumption be made, as it generally unwisely is, that ovulation occurred 2 weeks after the LMP, then the fetus would have been 10 postovulatory weeks of age at the time of the accident. This seems likely, but only in view of the circumstance that the birth, which had, on the basis of the LMP, been estimated for 24 September, actually took place only four days later, on 28 September.

It has been mentioned that the normal tibia appears and chondrifies at 6 postovulatory weeks and develops a bony collar by 8 weeks. Hence it can be seen that a failure of the tibial primordium to undergo these changes would have to have been determined several weeks before an accident that occurred at 10 weeks. However, other possibilities do remain, namely, that tibial development could have been arrested from the time of the accident, or that regressive changes might have commenced from that time.

The paradoxical limb in question, however, presented not only deficiency but duplication. Because the metatarsals appear and

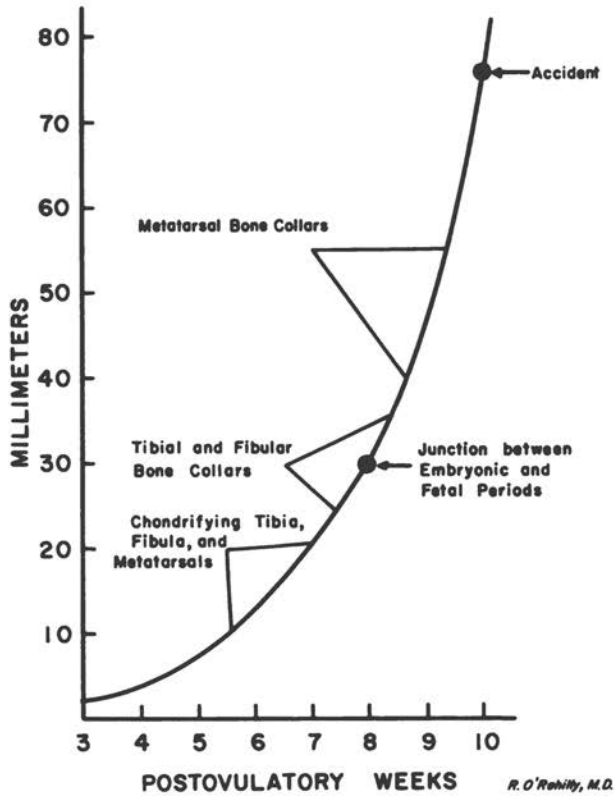

FIGURE 3 Case of tibial hemimelia and preaxial polydactyly. Graph of CR length to illustrate the sequence of certain skeletal events in the normal development of the leg and foot, and the time of an automobile accident which was unjustifiably claimed to have caused tibial hemimelia and preaxial polydactyly. It can be seen that formation of the relevant skeletal elements in mesenchyme (not shown), cartilage, and even bone, takes place largely within the embryonic period proper, whereas the accident in question occurred during the fetal period.

begin to chondrify by 7 postovulatory weeks and receive their bony collars by 9 weeks, it is clear that an increase in the number of metatarsals would have to have been determined several weeks before an accident that occurred at 10 weeks.

These embryological considerations were presented in court by Dr. Ernest Gardner and the writer. The case was awarded to the defendant.

REFERENCES

Forsthoefel, Paulinus F., "The Embryological Development of the Effects of Strong's Luxoid Gene in the Mouse," J. Morph., 113, 427-451 (Nov 1963).

Grant, Robert P., "The Embryology of Ventricular Flow Pathways in Man," Circulation, 25, 756-779 (May 1962).

Gruenwald, Peter, "Malformations Caused by Necrosis in the Embryo," Amer. J. Path., 34, 77-103 (Jan-Feb, 1958).

Hovelacque, A., and R. Noel, "Processus embryologique de l'absence congénitale du Tibia," C. R. Soc. Biol., 88, 577-578 (1923).

Zwilling, Edgar, and Jean F. Ames, "Polydactyly, Related Defects and Axial Shifts--a Critique," Amer. Naturalist, 92, 257-266 (Sept-Oct, 1958).

Control of Growth Patterns in Limb Development

JOHN W. SAUNDERS, JR., Ph.D.
Marquette University
Milwaukee, Wisconsin

The remarks in this paper are addressed to aspects of vertebrate limb development not usually treated in medical texts. They deal with factors underlying the morphological changes that occur during early stages of limb development. Because of their very nature these factors can be elucidated only by experimentation of a kind impossible to carry out on human material. Moreover, such delicate operative procedures are required that at present they cannot readily be practiced on the embryos of any higher mammals. The chick embryo, however, is quite amenable to operation; one simply makes a hole in the shell and, under the binocular dissecting microscope, intervenes microsurgically on the embryo lying atop the yolk.

There is no reason for us to think that the development of the limb in the chick embryo is different in principle from that of the human embryo. It arises from the body wall in similar fashion and produces an appendage constructed according to the same basic

plan. In the x-ray photograph of a chicken wing reproduced in Figure 1, the bones of the upper arm and forearm are easily recognized as homologous to those of corresponding levels of the human arm. The carpals and metacarpals are also homologous. They are, however, greatly reduced in number by the fusion of the embryonic rudiments, and the hand has only the second, third, and fourth digits.

THE NORMAL PATTERN OF LIMB GROWTH

At 72 hours of incubation the chick embryo is roughly equivalent in development to the 4-week human embryo. At this time, bulges in the body wall may be recognized as the primordia of the anterior and posterior appendages, respectively. In Figure 2 the outlines of the limb swellings are drawn with a heavy line; this represents the apical ectodermal thickening, or ectodermal ridge, to which Dr. Ronan O'Rahilly earlier directed our attention in a particular way. This ridge is a very important structure, and its role in determining the growth and form of the limb will occupy us considerably in what follows.

In the period between 72 and 96 hours of incubation (about 4 to 5 weeks in the scale of human development), the limb buds enlarge

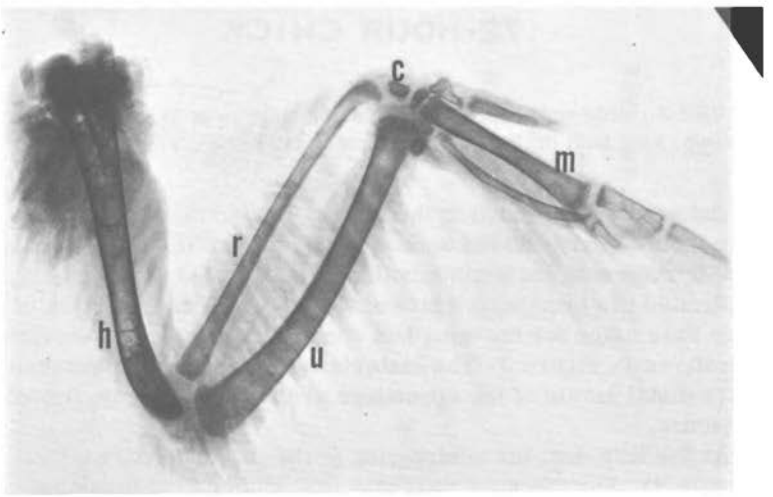

FIGURE 1 X-ray photograph of the wing of the juvenile fowl. h, humerus r, radius; u, ulna; c, carpal; m, metacarpal. (Modified from Saunders, et al., 1958.)

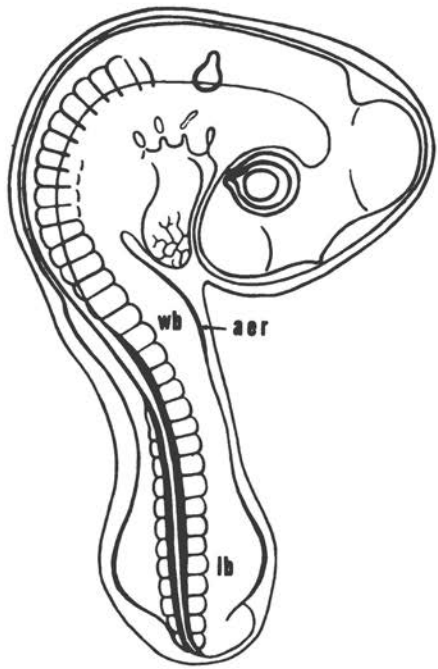

FIGURE 2 Schematic outline of the chick embryo at 72 hours of incubation. wb, wing bud; lb, leg bud; aer, apical ectodermal ridge.

as flat paddles appended to the body wall. During this period one may insert finely divided carbon particles into the limb-bud tissues and, by following their distribution in subsequent development, determine the prospective fate of tissues from each portion of the bud. Fate maps for the wing bud constructed from data so obtained are shown in Figure 3. The materials for forming the successively more distal levels of the appendage are laid down in proximodistal sequence.

At the fifth day, the sculpturing of the limb contours begins (Figure 4). For the most part this is accomplished by differential growth, but also contributing are localized zones of cataclysmic cellular degeneration. The degree to which necrosis is involved as a normal process in limb development varies from one vertebrate to another, but in all forms examined by the writer, or for

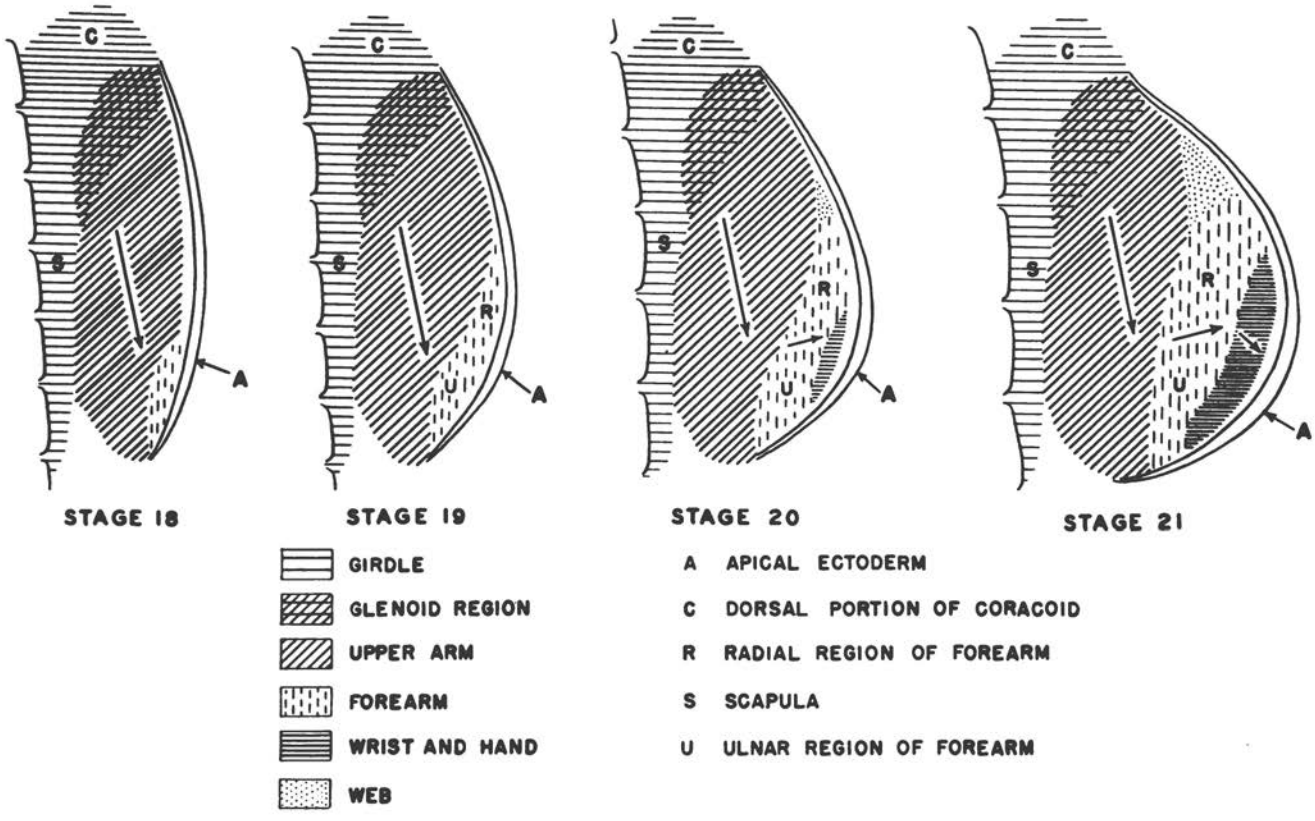

FIGURE 3 Fate maps for the chick wing bud constructed from carbon marking data. Numbers refer to stages of the Hamburger-Hamilton seriation of 1951. (Modified from Saunders, 1948.)

FIGURE 4 Sculpturing of the contours of the wing (left) and leg (right) primordia of the chick embryo. Numbers to right of each column are Hamburger-Hamilton stages. Stage 24 occurs at approximately 4 days of incubation; stage 33 at about 9 days. Zones of massive necrosis are shown by stippling.

which he has found reports, death appears consistently in the interdigital tissues as the contours of the fingers and toes emerge from the hand plates and foot plates. In the duck, necrosis in tissues between toes, which are webbed, is considerably reduced!

It is tempting to suggest that some cases of congenital soft-tissue syndactyly result from a failure of death in tissues inter-

vening between the digits rather than from failure of differential growth. This explanation is suggested by observations that the vital dye, Janus green, which stains the mitochondria, and the chemical, acriflavine [observations of Mr. John Fallon, at the Marquette University laboratory; (also Deleanu, 1965)], which interferes with the metabolic transport enzymes of the mitochondria, bring about a failure of interdigital necrosis and subsequent soft-tissue syndactyly of the toes. Conceivably, however, failure of the interdigital clefts to form in these cases results, not from a failure of necrosis, but from altered growth patterns produced through effects of the administered agents on pathways of oxidative metabolism. Failure of cellular death to occur might have been only a concomitant event and not a causal one.

ECTODERM - MESODERM DIALOGUE

In cross section (Figure 5), the early limb bud shows a relatively homogenous mesodermal core covered with a simple ectodermal epithelium, which is thickened apically where the cells assume a pseudostratified columnar configuration. This is the apical ectodermal ridge noted above, and it has a profound developmental significance. If one excises the ridge microsurgically, the sequential formation of limb parts ceases abruptly, and the degree to which the proximal segments do develop varies according to the stage at which the operation was performed (Saunders, 1948). If, however, a limb bud be denuded of its dorsal and ventral ectoderm but provided with an apical ridge, it can develop quite normally (Gasseling and Saunders, 1961). If an extra ectodermal ridge is added to a limb bud, two appendages develop (Zwilling, 1956).

From these observations we conclude that the ectodermal ridge is an inductor of limb outgrowth, communicating to the mesoderm some message (in a form as yet unknown) that tells it to produce successively the materials for progressively more distal limb parts.

Whatever this inductive message may be, however, it is relatively nonspecific. Thus, it does not determine whether the appendage shall be of the anterior or posterior type: Leg-bud mesoderm provided with an ectodermal ridge of wing origin forms leg; reciprocally, wing-bud mesoderm forms wing when its outgrowth is induced experimentally by leg-bud ectoderm (Zwilling, 1955). Moreover, the action of the ridge is nonspecific with respect to the species source of the ectoderm. Thus, limb-bud mesoderm of

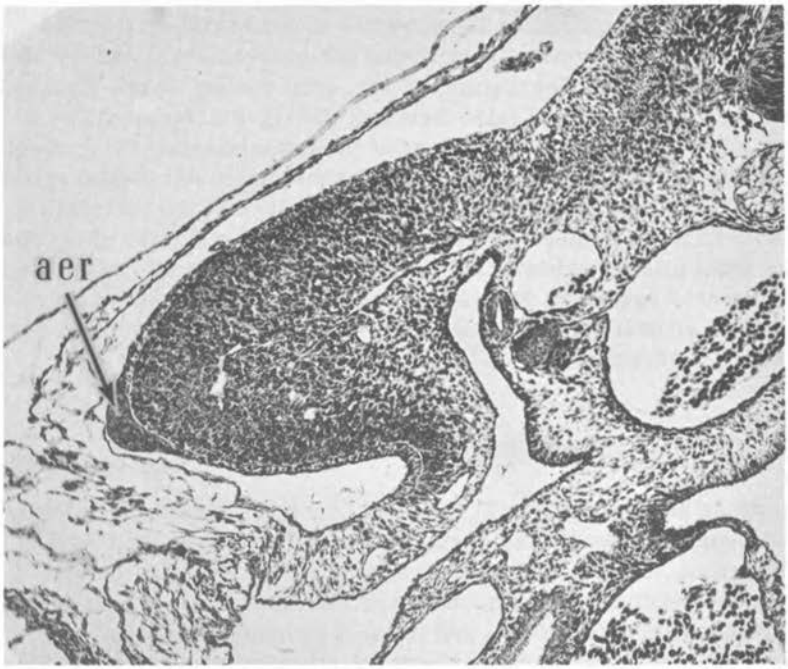

FIGURE 5 Cross section of the wing bud in a chick embryo at stage 18 (3 days of incubation). aer, apical ectodermal ridge. (Modified from Saunders, 1948.)

the chick or duck embryo, provided with ectodermal ridge of the other species, develops its successively more distal segment in perfectly good order (Hampé, 1956; Zwilling, 1959). Perhaps more dramatically, limb-bud tissue of the mouse will respond to induction by apical ridge of the chick in appropriate grafts (Cairns, 1965).

Thus we see that the ectodermal ridge "speaks a language" that the mesoderm can understand even though it may belong to a different order or class of vertebrate.

We have no reason to doubt that in the human embryo the ectodermal ridge of the limb bud issues a similar message to its own underlying mesoderm or that its message could also be understood by mesodermal tissues of other higher vertebrates.

Message traffic between ectoderm and mesoderm does not flow in one direction only. That mesoderm also communicates to the ectoderm is revealed in experiments designed to analyzé the origin of the apical ectodermal ridge. If prospective wing-bud mesoderm

from one embryo be grafted to a wound in the flank of another in such a way that flank ectoderm must regenerate over it, the flank ectoderm will form an apical ectodermal ridge and this, in turn, will induce the outgrowth of the mesoderm to form a complete supernumerary limb at the flank level. This result occurs only if the host and donor embryos are of at least stage 11 (a 10- or 11-somite embryo) and no older than stage 17 (wing bud just beginning to form) at the time of operation. Within this range of stages, any age combination of host and donor can be effective (Reuss and Saunders, 1965). From this we conclude that from stages 11 through 17, prospective flank ectoderm as well as prospective wing ectoderm is capable of making an ectodermal ridge and that it does so in response to inductive action exercised by the mesoderm. Moreover, the capacity to induce this response in a competent ectoderm is possessed by limb-bud mesoderm only during the same stages, namely 11 to 17; after this, it is lost.

The loss of its inductive voice, however, does not signal that the mesodermal contribution to the "conversation" is finished. Some years ago Zwilling showed that after the ridge is formed other factors in mesoderm are required in order that the ridge may remain thick and inductively active. These factors (apical ectoderm maintenance factors) are apparently located chiefly postaxially where the ridge is thicker and whence the major part of limb outgrowth occurs (Figure 6a). Dr. Zwilling's evidence for this view speaks eloquently in its own behalf in his original papers and in a number of reviews so that it is not necessary to repeat it here (Zwilling, 1961).

At Marquette University, however, as a result of experiments in which the apex of the wing bud was reversed on its own stump

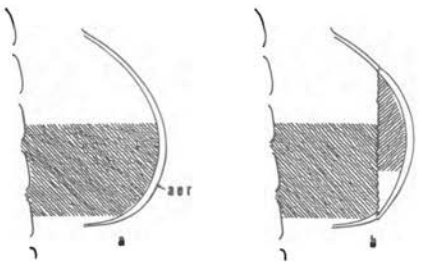

FIGURE 6 a. Distribution of the presumed apical ectoderm maintenance factor is shown for the chick wing bud at stage 21. b. Effect on the distribution of this factor of reversing the apex of the bud. aer, apical ectodermal ridge.

(Figure 6b), the writer has obtained verification of his hypothesis. Under these circumstances duplicate wing tips mirror-twinned in the radial plane formed in reversed dorsoventral orientation (Figure 7) from the reversed apex. This is interpreted to indicate that a "maintenance factor" from the postaxial portion of the stump is transmitted into the "factorless" (originally preaxial) reversed mesoderm. There it acts on the overlying ridge ectoderm, causing it to thicken and induce mesodermal outgrowth (Saunders et al., 1958).

Possibly the factor eventually becomes stabilized or is produced in appreciable quantities in the reversed preaxial mesoderm because, if the apex is left in the reversed position for as long as 14 hours and then restored to its original orientation, twin limb tips are formed just as in cases wherein the reversal is permanent, except that they are normally oriented dorsoventrally. If the restoration to normal orientation is made in less time, however, only a single limb tip usually develops.

The maintenance factor also has the property of being trans-

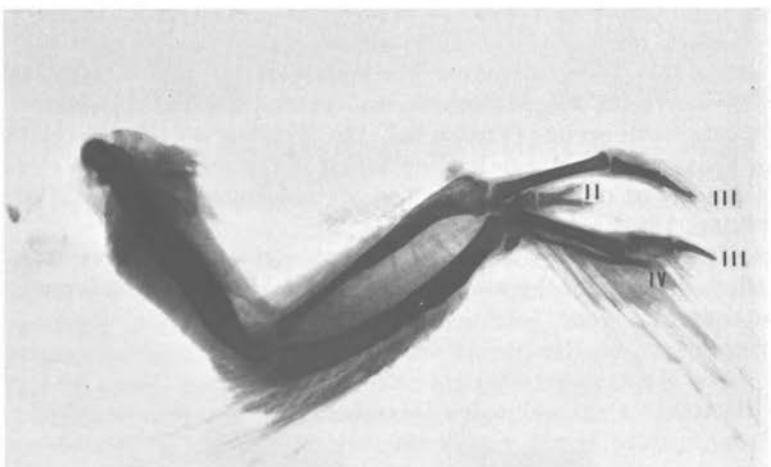

FIGURE 7 Mirror-twinning of the hand and distal portion of the forearm resulting from reversal of the apex of the wing bud in the chick embryo (cf. Figure 6). Note that digit II, twinned, lies in the mirror plane between rays III of the anterior and posterior hands, respectively. Metacarpal and phalanx IV are in the x-ray shadow of metacarpal III of the anterior hand. (Modified from Saunders and Gasseling, 1959.)

mitted through millipore filters, which bar cell contact (Figure 8); thus the factor must be diffusible, yet, if it is diffusible, there must be restraints on the direction in which it may move, otherwise it would be uniformly distributed throughout the limb (Saunders and Gasseling, 1963). This is a most puzzling situation, indeed; but for the present no solution is in view.

GENERAL CONCLUSIONS

The results herein reviewed clearly show that in the development of the vertebrate limb there is an ectoderm-mesoderm dialogue in which three messages are communicated: First, a mesodermal message, to which ectoderm responds by making an apical ridge, is apparently transmitted only during very early limb-bud stages (stages 11 and 17 in the chick embryo); the ectoderm is competent to respond to it only during the same period. Next, a message from the ectodermal ridge is sent to the underlying mesoderm, and the mesoderm responds by growing and laying down limb parts in normal proximodistal order. Finally, there is a mesodermal communication that instructs the ectoderm to remain thick and to continue inducing outgrowth in the mesoderm, the message source. The transmission and reception of these messages are essential aspects of limb development, but the natures of the messages remain to be clarified.

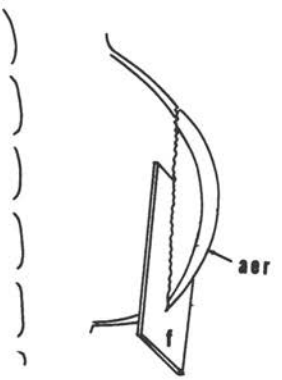

FIGURE 8 Method of interposing millipore filter between the postaxial portion of the wing-bud stump and the reversed apex. aer, apical ectodermal ridge; f, filter. (Modified from Saunders and Gasseling, 1963.)

REFERENCES

Cairns, J. M., "Development of Grafts From Mouse Embryos to the Wing Bud of the Chick Embryo," Develop. Biol., 12, 36-52 (1965).

Deleanu, M., "Toxic Action upon Physiological Necrosis and Macrophage Reaction in the Chick Embryo Leg," Rev. Roumaine Embryol. Cytol., 2, 45-56 (1965).

Gasseling, M. T., and John W. Saunders, "Effects of the Apical Ectodermal Ridge on Growth of the Versene-Stripped Chick Limb Bud," Develop. Biol., 3, 1-25 (1961).

Hamburger, V., and H. L. Hamilton, "A Series of Normal Stages in the Development of the Chick Embryo," J. Morph., 88, 49-92 (1951).

Hampé, A., "Influence de la calotte épidermique du bourgeon de patte de poulet sur la formation d'articles distaux," C. R. Soc. Biol., 150, 1671-1673 (1956).

Reuss, C., and John W. Saunders, "Inductive and Axial Properties of the Prospective Limb Mesoderm in the Early Chick Embryo," Amer. Zool., 5, (Abstract) (1965).

Saunders, John W., "The Proximodistal Sequence of Origin of the Parts of the Chick Wing and the Role of the Ectoderm," J. Exp. Zool., 108, 363-404 (1948).

Saunders, John W., and M. T. Gasseling, "Effects of Reorienting the Wing-Bud Apex in the Chick Embryo," J. Exp. Zool., 142, 553-570 (1959).

Saunders, John W., and M. T. Gasseling, "Trans-Filter Propagation of Apical Ectoderm Maintenance Factor in the Chick Embryo Wing Bud," Develop. Biol., 7, 64-78 (1963).

Saunders, John W., M. T. Gasseling, and M. David Gfeller, "Interactions of Ectoderm and Mesoderm in the Origin of Axial Relationships in the Wing of the Fowl," J. Exp. Zool., 137, 39-74 (1958).

Zwilling, Edgar, "Ectoderm-Mesoderm Relationship in the Development of the Chick Embryo Limb Bud," J. Exp. Zool., 128, 423-438 (1955).

Zwilling, Edgar, "Interaction Between Limb Bud Ectoderm and Mesoderm in the Chick Embryo, II. Experimental Limb Duplication," J. Exp. Zool., 132, 173-187 (1956).

Zwilling, Edgar, "Interaction Between Ectoderm and Mesoderm in Duck-Chicken Limb Bud Chimaeras," J. Exp. Zool., 142, 521-532 (1959).

Zwilling, Edgar, "Limb Morphogenesis," Advances Morph., 1, 301-330 (1961).

The Contribution of Histochemistry to Our Understanding of Limb Morphogenesis and Some of Its Congenital Deviations

JEAN MILAIRE, Ph.D.
Professor of Anatomy
University of Brussels
Brussels, Belgium

The great difficulties that have continued to impede the direct experimentation of mammalian embryos by microsurgical methods led the writer, about 10 years ago, to approach the problem of limb morphogenesis in the rat by standard histochemical techniques (Milaire, 1956).

By visualizing the precise localization of several chemical constituents of the developing cells and also by detecting some of their enzymatic properties, it was reasonable to expect that by such methods some dynamic aspects of the morphogenetic events involved in development would be identified. The real purpose was, in fact, to learn to what extent the fundamental ectoderm-mesoderm interactions recently demonstrated in the chick embryo also contributed to the formation of a mammalian limb.

At first sight, the histochemical observations presented the appearance of an intricate picture of various regional properties showing minor changes from stage to stage. However, when a

closer series of graded stages was studied, and particularly when precise correlations could be established between the structural and the histochemical changes, the meaning of the observations was greatly clarified. It gradually appeared, for example, that such enzymatic activity temporarily present in the ectoderm preceded or accompanied a rise in the growth of the underlying mesoderm. It appeared also that most of the histochemical changes taking place at later stages in the still undifferentiated mesoderm might be considered the earliest signs of various tissue differentiations.

It became progressively evident that, even if their biochemical significance still remained obscure, the histochemical properties of developing rudiments still can provide various kinds of interesting information about the developmental mechanisms, if constant attention is paid to the morphological changes occurring simultaneously in the corresponding areas.

This first impression was later confirmed by the results of the more detailed histochemical studies of limb morphogenesis in the mouse and the mole (Milaire, 1963) and more recently on the chick embryo (Milaire, in press).

Among the various observations made in mammalian embryos, a great number may be considered suggestive evidence of the inductive interactions between the mesoderm and the ectoderm. Others provide insight into the early organization and later differentiation of the various preskeletal, premuscular, and preconnective rudiments.

It is patently dangerous to interpret microscopic observations in terms of developmental mechanisms. Thus it is particularly necessary to compare the histochemical aspects of limb development observed in various mammals with those observed in the chick, one of the rare species in which a great deal is known about the morphogenetic factors involved. In addition, similar histochemical studies of limb morphogenesis have been made on different strains of mice affected by congenital limb abnormalities (Milaire, 1962, 1965). In many cases, the observations made improved or confirmed our previous interpretations of the normal histochemical features and also threw some light on the morphogenetic modifications responsible for the final malformations.

METHODS

Each histochemical technique has been performed on serial sections of the isolated limb buds or on transverse sections of the whole

embryo at very early stages. The orientation of the sections varies according to the stage considered and also according to the limb parts to be demonstrated. The early limb buds are studied either on transverse sections of the trunk region (Figure 1a) or on frontal

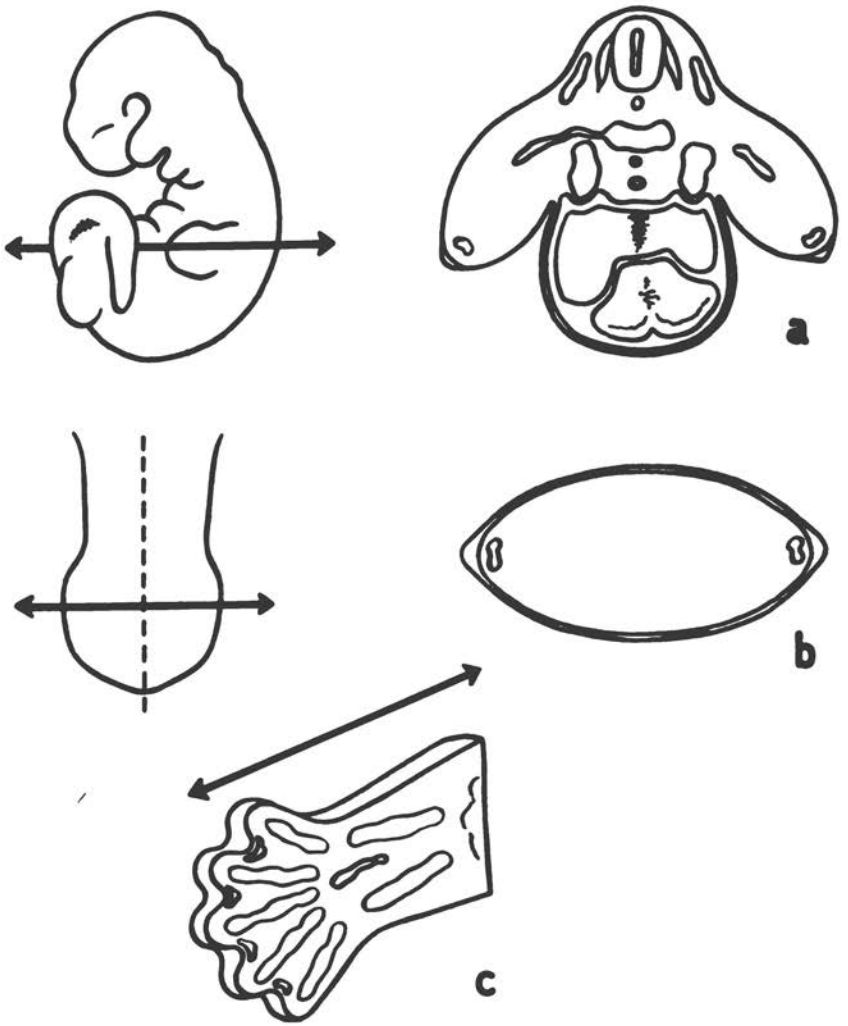

FIGURE 1 Orientation of the sections of early limb buds: a. Transverse sections through the forelimb buds in situ. b. Transverse sections of the isolated limb bud; the plane of sectioning is perpendicular to the limb-bud axis. c. Frontal sections of the isolated limb bud; the plane of sectioning is parallel to the ventral surface of the foot plate.

TABLE 1 Histochemical Demonstration of Dephosphorylating Enzymatic Activities

Substance or Enzyme Demonstrated	Fixative	Method	Main Localization in the Developing Limb Buds	Particular Advantages of the Method
RNA	Serra fixative	Unna-Brachet staining (pyronine-methyl green)	• Proliferating cells • Thickened areas of the undifferentiated ectoderm • Early precartilaginous and premuscular blastemata	• Demonstration of the finest structural changes • Very selective demonstration of the degenerating cells
Glycogen	Pasteels Leonard fixative	PAS	• Local decrease of metabolic activities which usually precedes degenerative phenomena • Early precartilages in the mole • Ossification centers	
Mucopoly-saccharides	id.	id. after salivary digestion	• Exocellular cartilaginous substance • Basement membranes	
Alkaline phosphatase (pH 9.2)	Cold absolute ethanol	V. Kossa-Barger Silver method Substrate: Calcium glycerophosphate	• Thickened areas of the undifferentiated ectoderm • Morphogenetically active parts of the undifferentiated mesoderm in the mole • Early stages of the transformation of the mesoderm into perichondrium and connective tissue • Ossification centers	

Acid phosphatase (pH 5)	id.	Gomori Lead method Substrate: Sodium glycero-phosphate	• Thickened areas of the undifferentiated ectoderm • Prearticular areas of the mesoderm	• Very selective demonstration of the degenerating cells
Tri- and diphosphate Mononucleotide Phosphohydrolase (pH 7.2)	id.	Wachstein-Meisel Lead method Substrates: ATP-ADP UTP-UDP ITP - IDP	• Thickened areas of the undifferentiated ectoderm • Somatopleural mesoderm in the chick embryo • Early digital mesoderm • Perichondrium • Prearticular mesoderm • Vascular endothelia	• Nice demonstration of the vascular embryonic network
Monophosphate Mononucleotide Phosphohydrolase (pH 7.2)	id.	Wachstein-Meisel Lead method Substrates: AMP-IMP	• The whole embryonic ectoderm • Preconnective areas of the mesoderm • Early limb-bud mesoderm in the chick embryo	

sections of the isolated rudiments, that is, sections parallel to their ventral surface (Figure 1c). At later stages, all limb buds are separated from the embryo and studied either on frontal sections (Figure 1c) or on transverse sections perpendicular to the proximodistal axis (Figure 1b). The frontal sections are convenient for studying the skeletal structures, while the transverse ones are more suitable for demonstrating the rudiments of soft tissues.

The technique was specifically modified for its application on embryonic tissue and for the practice of serial sections. A special comment must be made about the histochemical demonstration of the various dephosphorylating enzymatic activities (Table 1). Whatever the substrate or the pH of the incubating medium, the material was fixed in cold absolute ethanol and then dehydrated, embedded in paraffin, and sectioned. Each step of the enzymatic reaction was then performed on the nondeparaffinized sections, which were allowed to float freely at the surface of the various solutions. This procedure allows preservation of active enzymes in the tissues and permits a precise localization of the final product of the enzymatic activity in the sections.

Observations on Normal Limb Buds of the Mouse, the Rat, the Mole, and the Chick

Most of the following observations are common to the four species considered. Some properties, however, were found in only two or even one species. In spite of their more limited value, such particular features will be taken into consideration when their occurrence improves our understanding of the developmental mechanisms involved.

1. Origin and Properties of the Early Limb Bud

In all species studied, the onset of limb morphogenesis appears as a sudden increase of cell proliferation in the dorsal somatic mesoderm all along the trunk region. The resulting mesodermal cells raise the overlying ectoderm and form a lengthwise swelling extending from the cardiac area up to the cloacal region (Figure 2). [This structure was first described in the chick embryo by von Baer (O. Hertwig, 1906), who named it Wolff's crest.] A few hours later, the mesodermal growth proceeds actively at both extremes of the crest, giving rise to the anterior and posterior limb buds;

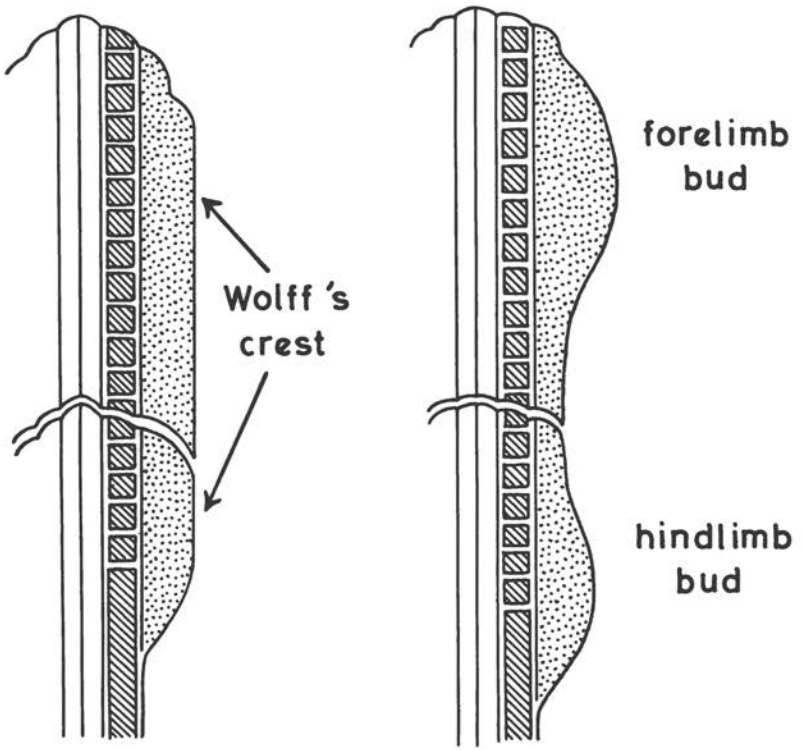

FIGURE 2 Wolff's crest (left) and early outgrowth of the limb buds (right).

simultaneously it decreases in the intermediate portion, which later will form the thoracic and abdominal walls.

From the histochemical point of view, a large amount of cytoplasmic RNA is already present in the thickened layer of somatic mesoderm before it starts proliferating (Figure 3). A high RNA content in the mesodermal cells remains the most common histochemical property of the presumptive limb constituents during the entire proliferating period (Figure 4). In the early stages, the mesodermal basophilia remains high in the intermediate part of the original Wolff's crest; however, it gradually decreases in this material as soon as it has formed. In the limb-bud areas, as long as new mesodermal cells are formed by the proliferating activities of the somatopleural layer, the overlying ectoderm keeps a common undifferentiated appearance and does not show any histochemical peculiarity (Figure 4). It is important to note that the growing activities of the somatic mesoderm do not start simultaneously all along the

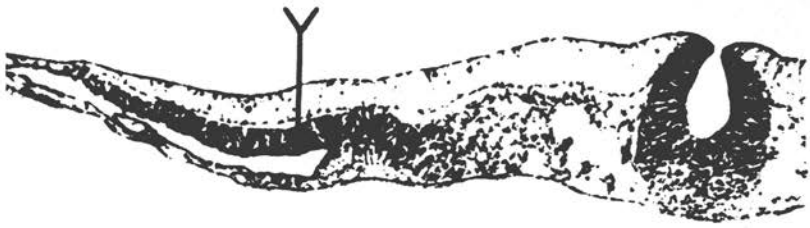

FIGURE 3 Chick embryo at stage 14 (Unna-Brachet staining): transverse section through the presumptive area of the hindlimb bud. The somatic mesoderm is thickened and provided with high RNA content.

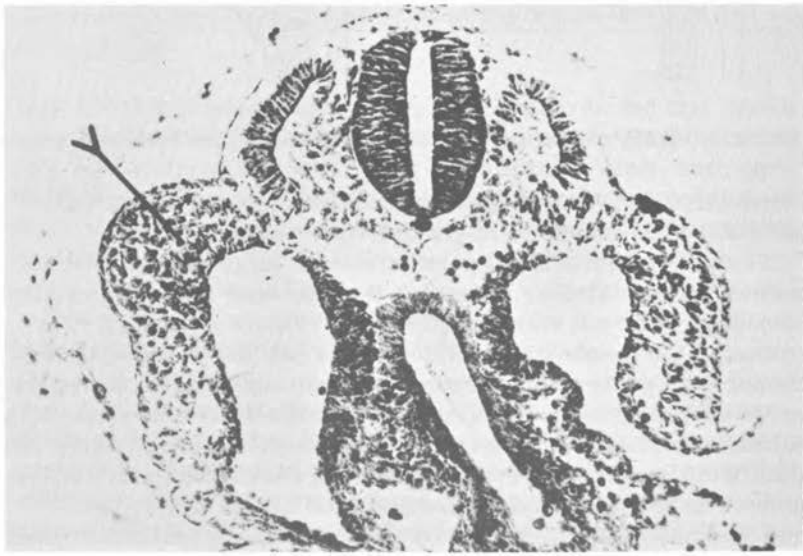

FIGURE 4 Mouse embryo at 9 days (Unna-Brachet staining): transverse section through the forelimb buds in situ. The limb-bud mesoderm is still originating from the dorsal somatopleura and is still rich with RNA. The limb-bud ectoderm is thin and undifferentiated.

trunk region. They first appear behind the cardiac area and thence proceed gradually in a cephalocaudal direction. Consequently, the anterior limb bud arises a few hours before the posterior one.

From these few observations, an initial conclusion, which fully agrees with the results of the experiment, is that the first demonstrable activities of limb morphogenesis take place in the mesoderm and do not involve any ectodermal change.

Two important questions, however, still remain unanswered: (1) Is the early formation of the limb-bud material the expression of intrinsic properties present in the somatic mesoderm or the result of some earlier extrinsic influence? (2) Are the limb formative properties uniformly distributed in the lateral mesoderm of the entire trunk region?

The experiments performed on Ambystoma by Detwiler (1933) and the more recent results on the chick embryo obtained by Chaube (1959) have shown that the presumptive limb mesoderm is endowed with limb properties at very early stages—for example, the gastrula stage for the amphibian, and stages 8 to 11 for the chick embryo. On the other hand, we know from the experimental results obtained by Kieny (1960) that the presumptive limb mesoderm of chick embryos is already determined with respect to its further transformation into wing or leg structures.

It was therefore interesting to explore these early stages from various histochemical points of view and to be particularly attentive to each regional peculiarity that might be demonstrative of a mesodermal specificity. Observations of that kind were made recently in sections of early chick embryos by detecting the activity of the AMP-phosphohydrolase. Although this reaction is absent in the corresponding region of mammalian embryos, it was demonstrated in the presumptive wing area of the chick at stage 16, near the early beginning of the proliferative period (Figures 5 and 6). Similar activity was observed a few hours later in the early mesoderm of the hindlimb bud where the reaction, however, is much weaker than in the wing area. It must be pointed out that at the same stages of the chick embryo the whole somatic mesoderm of the trunk region shows a uniform ATP-phosphohydrolase activity with no difference between the limb-bud areas and the intermediate region. In mammals, the same reaction characterizes the vascular endothelia only at the corresponding stages.

A last comment must be made about the behavior of the lateral trunk mesoderm interposed between the limb rudiments. In all species studied, this material loses its initial high concentration in RNA and becomes a loose mesoderm whose proliferating activi-

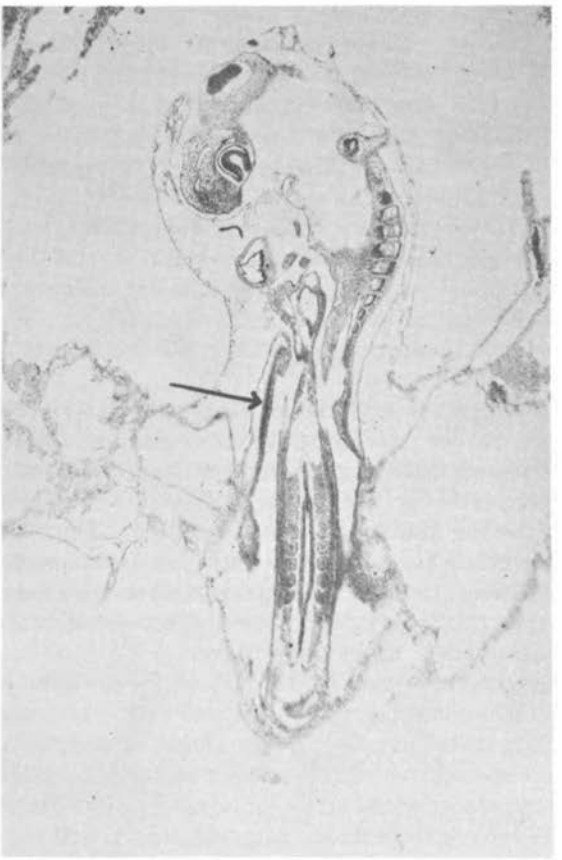

FIGURE 5 Chick embryo at stage 15, activity of AMP-phosphohydrolase: frontal section of the whole embryo. Strong enzymatic activity in the early forelimb bud mesoderm (arrow).

ties are very weak. In the chick embryo (but not in mammals), the Wolff's crest mesoderm further undergoes a sudden degeneration. As shown in Figure 7, the dying cells are characterized histochemically by the presence of large vacuoles containing RNA or DNA, and also by the strong acid phosphatase reaction demonstrable in most of these cytolytic vacuoles. The degenerating cells simultaneously lose their ATP-phosphohydrolase activity. Everything happens as if the Wolff's crest mesoderm suddenly were deprived of a hypothetical growth-stimulating factor. So far, however, there is no experimental evidence of the presence of such a factor in the origin of the limb-bud mesoderm.

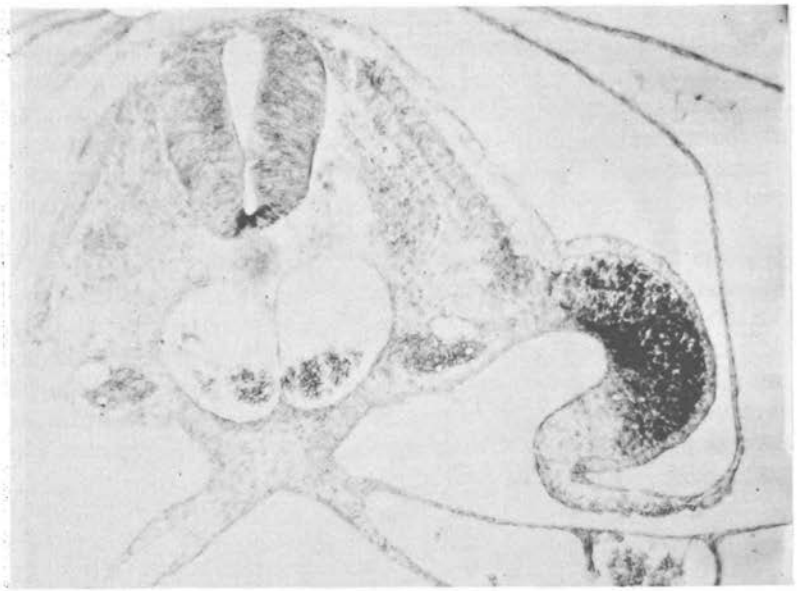

FIGURE 6 Chick embryo at stage 16: transverse section of the left forelimb bud in situ. Distribution of the AMP-phosphohydrolase activity in the limb-bud mesoderm.

2. The First Ectoderm - Mesoderm Interactions

It has been well established by experimental methods that one of the first events of limb morphogenesis is the occurrence of an inductive influence through which the young mesoderm induces the thickening of an apical ridge in the marginal portion of the overlying ectoderm. It is also known that the responsible mesodermal influence is stronger postaxially than preaxially and that the maintenance of the induced ectodermal ridge requires a continuous stimulation exerted by the same mesoderm that was its first inductor. It has been demonstrated that the mesodermal maintenance factor is a diffusible substance capable of passing through a millipore filter (Saunders and Gasseling, 1963). Nothing is known, however, about its chemical nature or about the metabolic properties of the mesoderm in which the substance has its origin.

In mammals, the only structural indication of the possible occurrence of such a mesodermal influence is the existence of minor differences in the thickness of the ectodermal ridge on both sides of the limb-bud axis. As in the chick embryo, the ridge is thicker

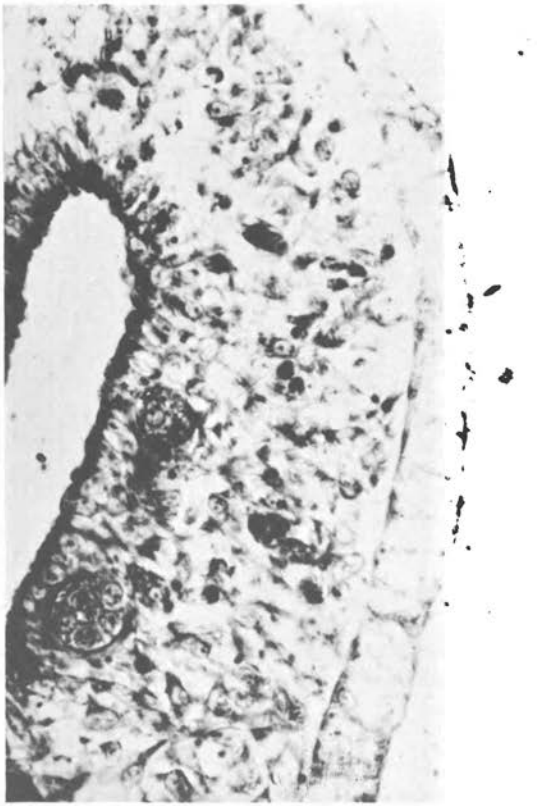

FIGURE 7 Chick embryo at stage 17: transverse section of the lateral body wall lying between both pairs of limb buds. Many cytolytic vacuoles can be seen in the mesoderm (Unna-Brachet staining).

postaxially than preaxially. Histochemical analysis has provided two kinds of interesting information about the early interactions between the ectoderm and the mesoderm. It has shown that the apical ectodermal ridge is not the only part of the ectoderm to be modified by the mesodermal influence. Analysis has also shown that the several mesodermal properties were found asymmetrically distributed between the preaxial and postaxial territories. These properties may reasonably be considered histochemical evidences of the mesodermal maintenance factor.

In all species studied during a limited developmental period, definite regions of the limb-bud ectoderm undergo the same structural and histochemical changes as soon as the mesoderm has been

laid down. Morphologically, the cells of the basal layer increase in size, and additional cells appear in this layer either by mitotic proliferation or by the transformation and further migration of peridermal cells toward the basement membrane (Figure 8). From the histochemical point of view, the modified ectodermal cells acquire large amounts of RNA in their inner pole, that which comes to lie close to the basement membrane, with strong cytoplasmic reactions of acid and alkaline phosphatases and of ATP-phosphohydrolase.

In the early mouse forelimb bud, the first ectodermal changes take place over the entire ventral surface of the bud, as shown in Figure 9. Thus, at these early stages, there is no evidence of an apical ectodermal ridge. This latter structure begins forming a few hours later at the postaxial extremity of the marginal border, and this morphological change is preceded and later accompanied by a remarkable increase of all histochemical properties previously

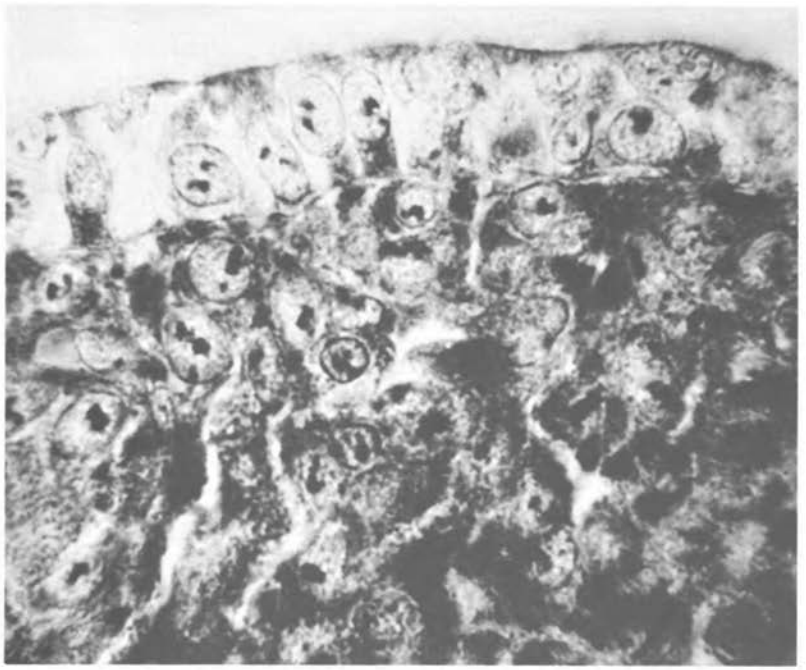

FIGURE 8 Mouse embryo at 9-1/2 days: ventral part of the hindlimb bud sectioned transversely in situ. Detail of thickening of the ventral ectoderm (Unna-Brachet staining). A large amount of cytoplasmic RNA is present in the mesoderm and in the inner pole of the ectodermal cells.

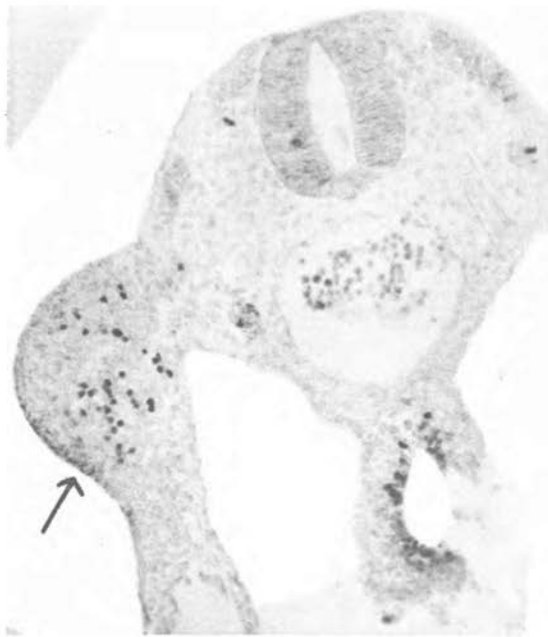

FIGURE 9 Mouse embryo at 9 days, acid phosphatase reaction. Strong enzymatic activity in the ventral-thickened ectoderm of the forelimb bud (arrow).

present in the ectodermal cells. The caudocephalic gradient presiding over the formation of the ectodermal ridge can be demonstrated by comparing either the preaxial and postaxial regions of the same limb bud, or the same area in different rudiments at successive developmental stages. The former demonstration is illustrated in Figure 10, which shows the distribution of RNA in the mouse hindlimb bud, and the latter is illustrated in Figure 11. At still later stages, the histochemical properties gradually decrease in the ventral ectoderm and persist at a high level in the apical ectodermal ridge (Figure 12).

Although these ectodermal modifications occur similarly in mammalian and chick embryos, their chronological sequence differs slightly in the two groups of vertebrates. The ectodermal ridge forms earlier in the chick limb bud than in the mammalian rudiment,

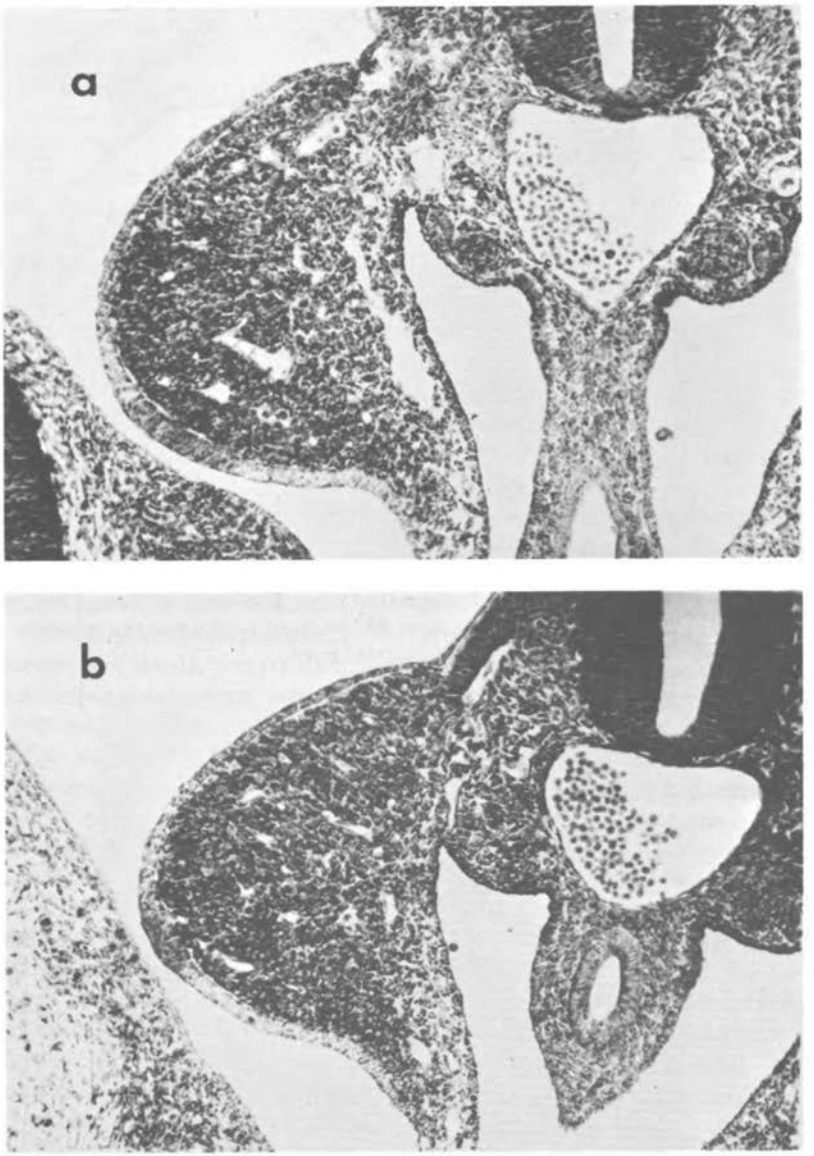

FIGURE 10 Two transverse sections through the 10-day mouse hindlimb bud (Unna-Brachet staining): a. Preaxial part of the limb bud; RNA is concentrated in the limited portion of the ectoderm, which later will give rise to the apical ectodermal ridge. b. Postaxial part of the limb bud; the corresponding region of the limb-bud ectoderm has begun its transformation into a crestlike structure.

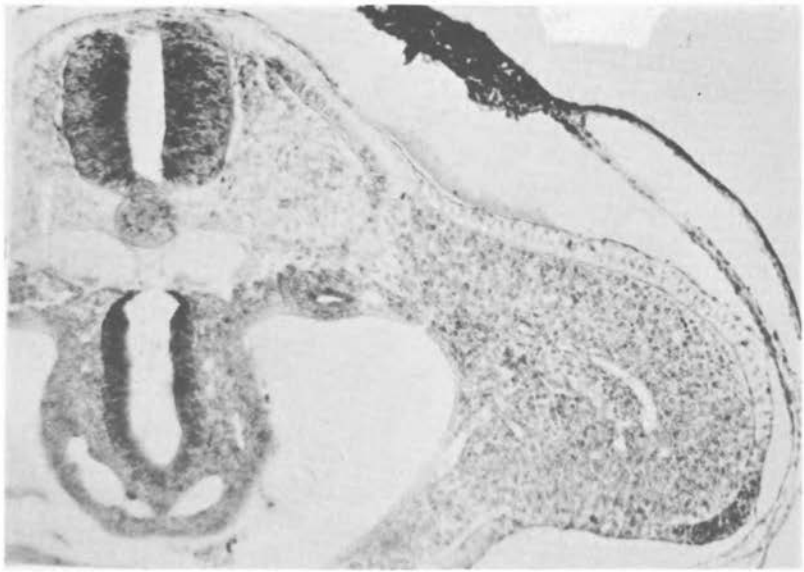

FIGURE 11 Chick embryo at stage 18: transverse section through the left hindlimb bud in situ. Strong acid phosphatase activity in the recently formed apical ectodermal ridge (postaxial part).

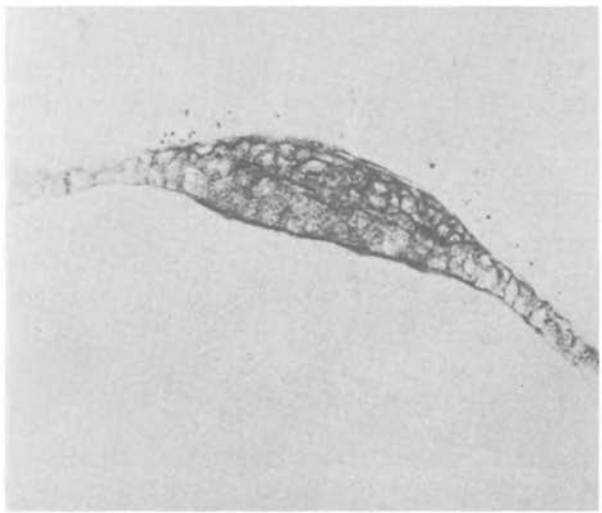

FIGURE 12 Mole embryo, apical ectodermal ridge of the forelimb bud: selective activity of alkaline phosphatase in the ridge.

and the histochemical properties of the ventral ectoderm remain present in mammals during a longer developmental period (Figure 13). In both groups, however, the ventral ectoderm remains thicker than the dorsal ectoderm until advanced stages of the limb-bud outgrowth.

If one looks at the maps of the presumptive territories in chick limb buds, it may be noted that the ectodermal ridge is already present when the mesoderm for the two proximal limb segments is laid down. At corresponding stages in the mouse embryo, the apical ectoderm has not yet acquired a crestlike appearance. Consequently, if the origin and determination of the proximal mesoderm in mammals require an ectodermal influence, it may be assumed that this role is played by the apical ectoderm before its transformation into an ectodermal ridge. This assumption is supported by the presence of selective histochemical properties in the apical area of the ectoderm long before its structural transformation (Figure 14). Such a consideration, of course, implies that morphogenetic properties appear very early in the thickened ectoderm, even before the ectoderm itself has had time to complete its full reaction to the mesodermal induction.

The same developmental period brings several changes in the mesoderm itself, providing clear evidences of the existence of asymmetrically distributed mesodermal properties.

First, there is a quantitative difference in the amount of basophilic mesoderm between the preaxial and postaxial halves of the limb bud, the postaxial area having more than the preaxial. This can be demonstrated at very early stages in frontal sections of the isolated rudiments. Secondly, there are at least two enzymatic activities that were found differently distributed on each side of the limb-bud axis. This is true for alkaline phosphatase in the limb buds of moles, as seen in Figure 15. The reaction is present in the postaxial area only. A similar distribution was found in the chick limb buds with respect to the AMP-phosphohydrolase activity. In the forelimb bud, this activity is stronger postaxially than preaxially; in the hindlimb bud, it is nearly absent in the preaxial mesoderm and largely distributed in the postaxial mesoderm (Figure 16).

The asymmetrical pattern of distribution of these enzymatic activities and the limited developmental period during which they occur strongly suggest that they might be histochemical expressions of the morphogenetic properties through which the mesoderm induces and later maintains the apical ectodermal ridge.

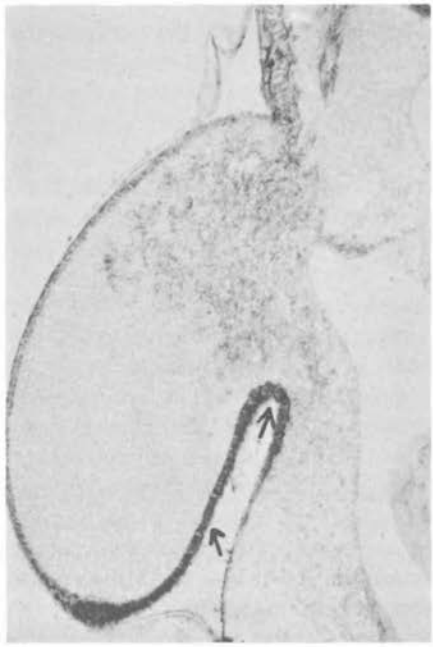

FIGURE 13 Mole embryo: transverse section through the right forelimb bud in situ. Strong alkaline phosphatase reaction in the ventral ectoderm (arrows) and in the proximal mesoderm.

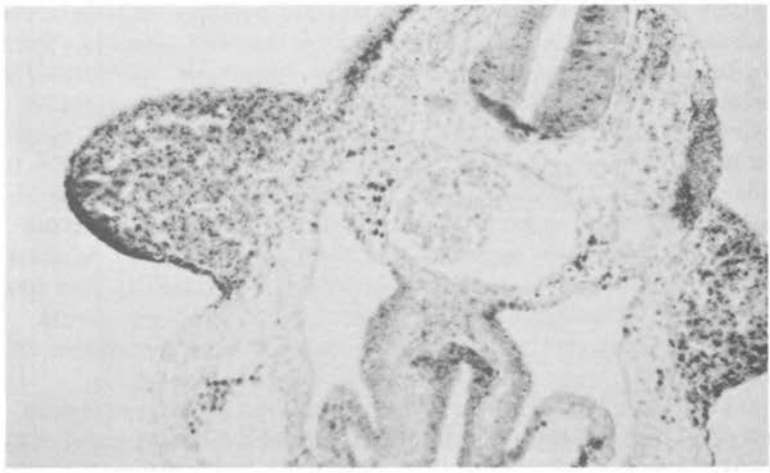

FIGURE 14 Mouse embryo at 9 days: transverse section through the right forelimb bud in situ. Strong acid phosphatase reaction in a limited ventro-apical portion of the limb-bud ectoderm, before its transformation into an apical ectodermal ridge.

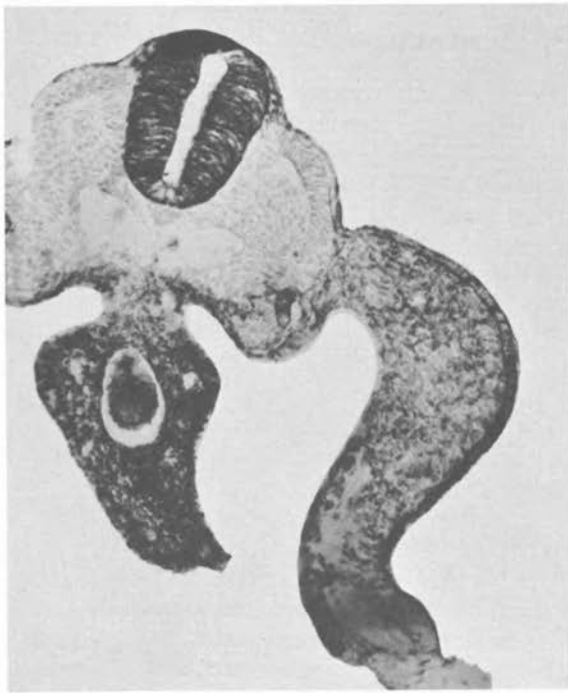

FIGURE 15 Early mole embryo: transverse section through the left forelimb bud (postaxial area). Strong activity of alkaline phosphatase in the ventral ectoderm and in the whole mesoderm. No such reaction is present in the mesoderm of the preaxial territories.

3. The Role of the Apical Ectodermal Ridge in the Formation of the Distal Limb Segment

Having considered the histochemical changes accompanying the early interactions between the mesoderm and the ectoderm, attention is now directed to the later histochemical evidences of the role played by the ectodermal ridge in the formation of the distal limb structures. The following data concern the mouse and mole embryos exclusively. The study of chick limb buds has not yet reached these advanced stages.

Looking at a transverse section of a 10-day mouse hindlimb bud (Figure 17), it is seen that the material involved in the forma-

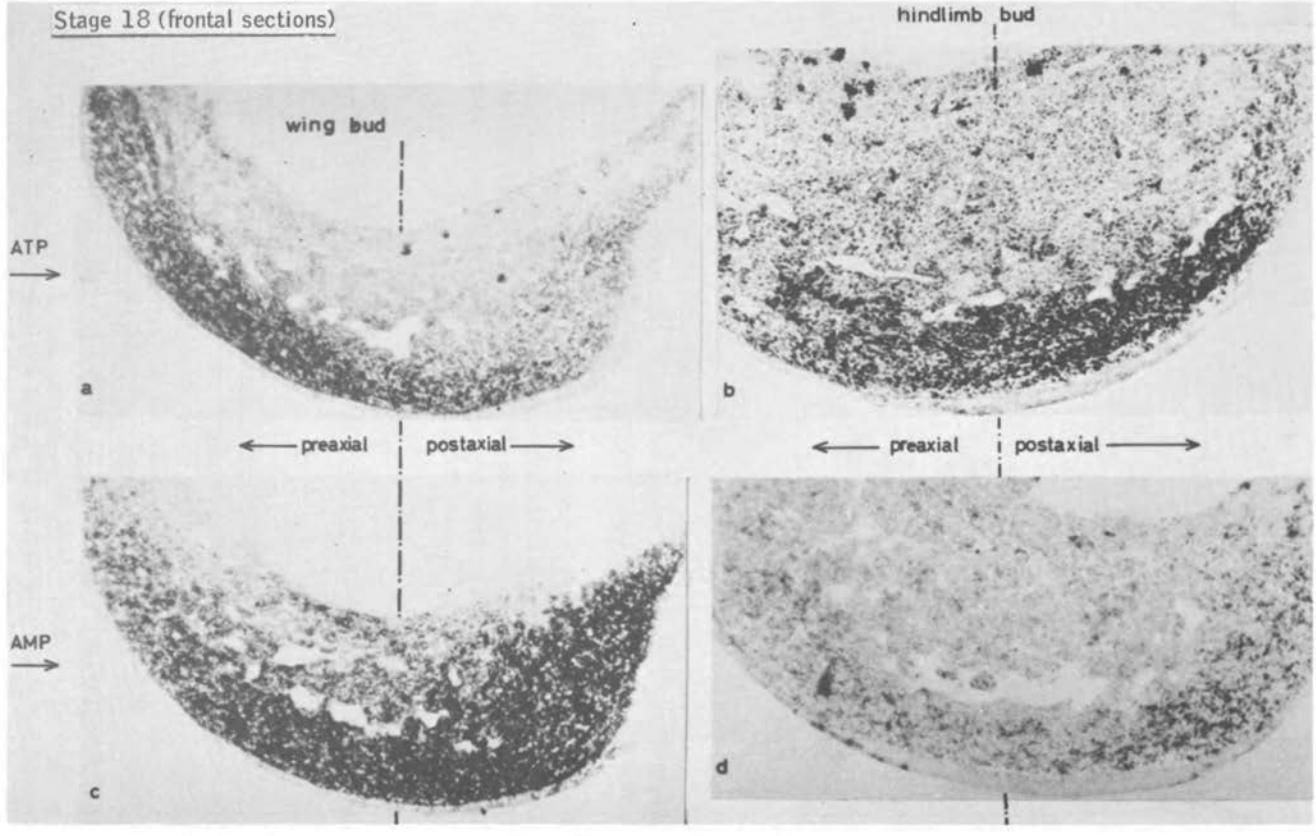

FIGURE 16 Chick embryo at stage 18: a, c, frontal sections through the wing bud; b, d, frontal sections through the hindlimb bud; a, b, distribution of the ATP-phosphohydrolase activity; c, d, distribution of the AMP-phosphohydrolase activity. In both rudiments, the mesodermal ATP-phosphohydrolase activity is uniformly distributed on both sides of the limb-bud axis, while the mesodermal activity of the AMP-phosphohydrolase is predominant postaxially.

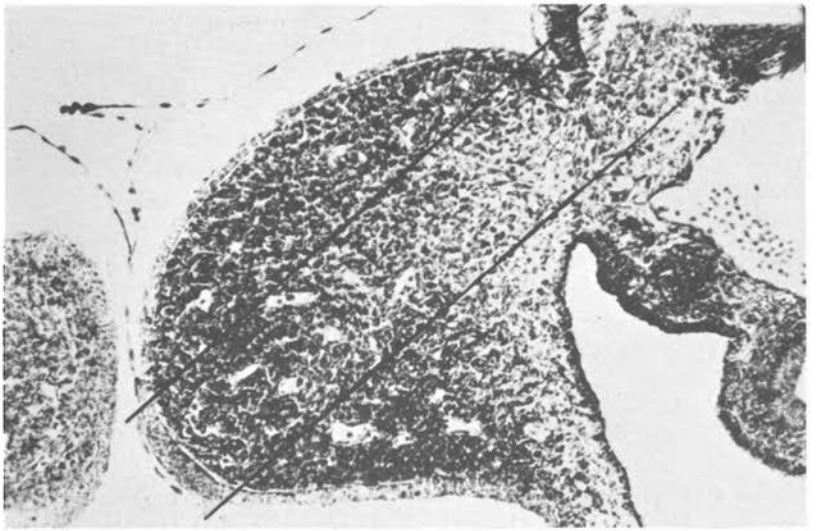

FIGURE 17 Mouse hindlimb bud in situ at 10 days (Unna-Brachet staining).

tion of skeletal structures is found in the central area facing the apical ectodermal ridge. This region has been approximately demarcated by two parallel lines passing through the dorsal and ventral borders of the ridge. The mesoderm lying outside these limits may be considered the presumptive material for soft tissue such as muscles, tendons, and connective tissue.

Examined on a frontal section, the central part of a 10-1/2-day mouse hindlimb bud appears to be divided into two different parts. The proximal two thirds of the rudiment is now covered by ordinary ectoderm and grows into the mesoderm, the early precartilaginous blastemata of the femur, the tibia, and the fibula, the last two mentioned being less condensed than the femur. These proximal territories have been determined at earlier stages, when the apical ectoderm has not yet undergone its transformation into a ridge-like structure. The distal third of the limb bud, a portion of which must necessarily be considered the presumptive foot, is composed of an undifferentiated mass of mesoderm covered distally with the apical ectodermal ridge. This structure has now reached the same thickness along its whole length, and it has also been carried distally by the outgrowth of the more proximal territories.

In spite of its uniform morphological appearance, the ectodermal ridge has undergone important cytochemical changes that can be related to the sudden occurrence of cell degeneration in its preaxial

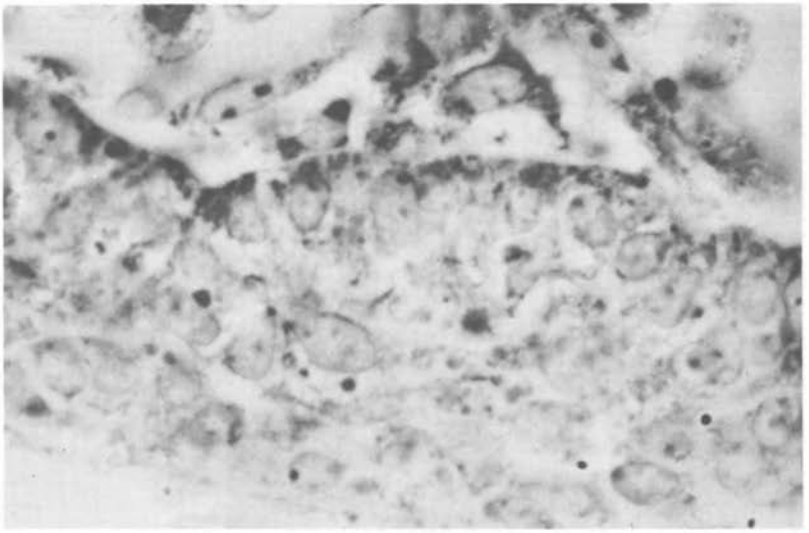

FIGURE 18 Mouse hindlimb bud at 10-1/2 days: frontal section of the apical ectodermal ridge in its preaxial portion (Unna-Brachet staining). Very small amount of RNA is present in the inner cells close to the basement membrane, and cytolytic vacuoles are visible in the more superficial cells.

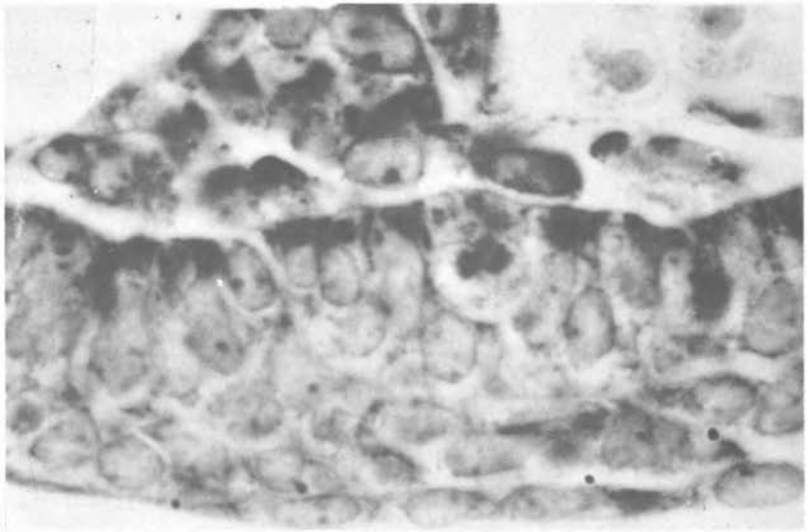

FIGURE 19 Mouse hindlimb bud at 10-1/2 days: frontal section of the apical ectodermal ridge in its postaxial portion. Large amounts of RNA are present close to the basement membrane, and all cells look healthy.

portion only. The most obvious change is a decrease of the RNA content in the basal cells (Figures 18 and 19). When compared with the postaxial part of the ridge, the preaxial portion shows much less RNA close to the basement membrane, and it contains a great number of dying cells. However, the preaxial involution of the apical ectodermal ridge causes a significant decrease in the alkaline phosphatase activity; conversely, the reaction of acid phosphatase increases in the same region as a consequence of the role played by this enzyme in cytolytic changes.

Such observations clearly demonstrate that a sudden redistribution occurs in the metabolic properties of the ectodermal ridge whose activities become predominantly postaxial. It is important to note that the resulting caudocephalic gradient appeared in the apical ectoderm and remains demonstrable in this structure up to the late 12-day stage, that is, up to the period when this structure recovers a normal thickness.

During the same period, the presumptive foot-plate mesoderm grows actively and gives rise to five radiated blastemata, including the presumptive material of the tarsal and metatarsal rudiments. It is remarkable to observe that all mesodermal changes involved in this matter occur as if they were the result of an ectodermal stimulation predominant in the caudal portion of the apical ridge. As shown in Figures 20 and 21, the mesoderm starts proliferating in the postaxial area, and this activity progresses slowly in a caudocephalic direction up to the 12-day stage. As soon as a definite amount of mesoderm has been laid down at a specific level along this caudocephalic progression, the more proximal mesodermal cells group together to form a radiated blastema including the presumptive tarsal and metatarsal elements of the corresponding digit. The fifth and fourth mesodermal columns appear at the early 11-day stage (Figure 20); the third column condenses about 10 hours later (Figure 21), while the second and first blastemata form at the early 12-day stage. In addition to the fact that the radiated blastemata are formed one after another from the fifth to the first, each of them also differentiates gradually in a proximodistal direction. The cellular condensation starts at the proximal extremity and proceeds distally. During a long period, all blastemata end distally in a common marginal layer of undifferentiated mesoderm. This material is the more recently laid-down mesoderm. It will contribute to the lengthwise growth of each radiated blastema.

In the formation of the first blastema in the extreme preaxial area of the foot plate, not only is this rudiment the smaller one, but its formation is preceded and later accompanied by important

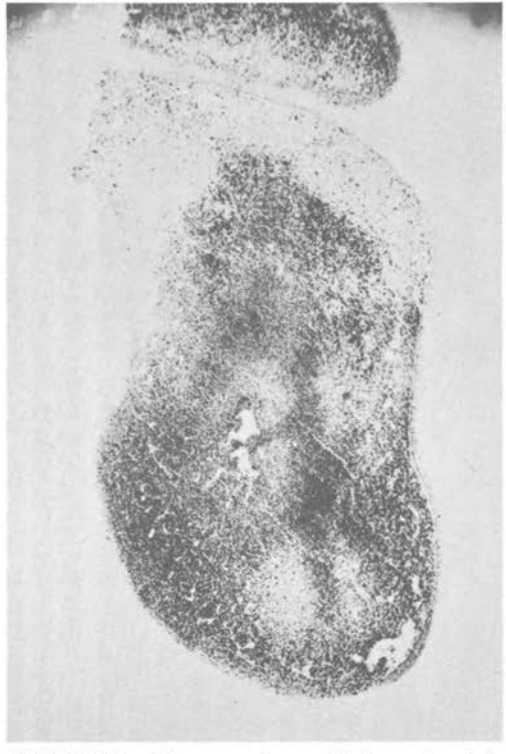

FIGURE 20 Mouse embryo, 11 days: frontal section through the right hindlimb bud (Unna-Brachet staining). The fourth and fifth radiated blastemata have formed in the postaxial area of the foot-plate mesoderm.

FIGURE 21 Mouse embryo, 11-1/2 days. The third radiated blastema is forming along the limb-bud axis; the second and first blastemata will condense at a later stage.

cytolytic changes occurring in the surrounding mesoderm (Figure 22). It was remarkable to observe such involutive changes in a region where the ectodermal ridge is supposed to exert a decreased morphogenetic influence. If we assume that the dying cells are those that would have contributed to the formation of an additional phalanx, the shortness of the first toe may be considered the result of weak morphogenetic properties present in the preaxial part of the apical ectoderm.

This first group of observations in the developing foot plate strongly suggests that the growth and probably also the early precartilaginous organization of the mesoderm are controlled by ectodermal influences predominant in the postaxial portion of the apical

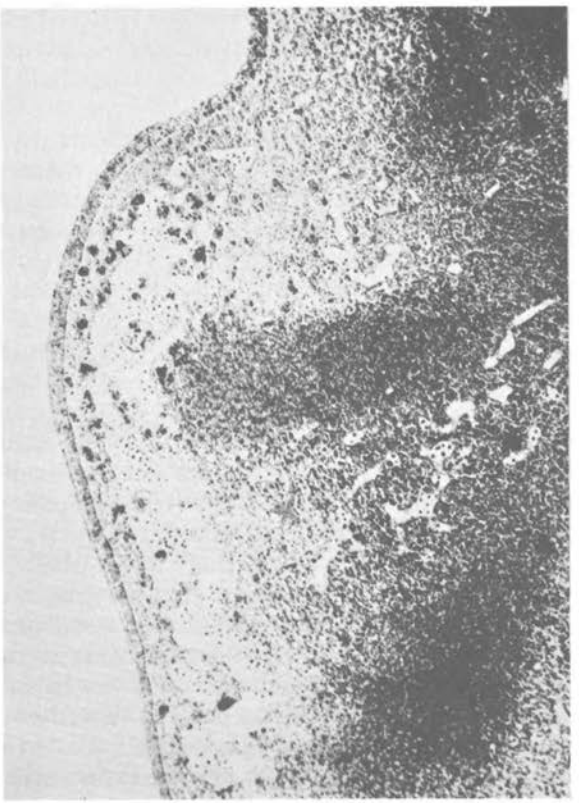

FIGURE 22 Mouse hindlimb bud at 12 days: preaxial part of a frontal section of the isolated rudiment (Unna-Brachet staining). Many dying cells are demonstrated in the preaxial mesoderm facing the first radiated blastema.

ridge. Further information is needed to explain why a heterogenous response is given by the mesoderm to a uniform (though asymmetrically distributed) ectodermal influence. In other words, it is still difficult to understand why the precartilaginous material of the distal segment is initially laid down as five separate units and not as a common precartilaginous plate; it is probably due to more intrinsic and still unexplored mesodermal properties involving competitive and regulative factors.

Considering now the later aspects characterizing the genesis of the digital material, histochemical observations in mammalian embryos strongly suggest that the last step of limb morphogenesis involves a sudden redistribution of the mesoderm-ectoderm relationships. During the entire period of digital outgrowth, everything happens as if the morphogenetic properties initially present in the whole apical ectoderm were selectively maintained at the tip of each digital bud and suppressed in the four intermediate interdigital areas.

As soon as the pentamerous system of radiating mesodermal columns has been formed, very selective necrotic changes occur simultaneously in the interdigital portions of the apical ectoderm and in the underlying mesodermal cells. These phenomena start postaxially in the fourth area (Figure 23) and later affect the other three interdigital regions. Soon after that, the enzymatic properties of the apical ectoderm disappear in the same interdigital regions, while more and more dying cells become demonstrable in the underlying marginal mesoderm (Figure 24). Conversely, at the tip of each digital bud, the apical ectoderm continues to demonstrate high concentrations of RNA (Figure 25) and shows a selective alkaline phosphatase activity. The maintenance of histochemical properties in the apical ectoderm of the digital buds remains unchanged up to the middle of the 13th developmental day, namely, long after the structural involution of the apical ridge into a two-layered ectodermal sheet. In the same period, the digital mesoderm continues to grow actively, and it successively gives rise to the precartilaginous rudiments of the proximal, middle, and distal phalanges. When all histochemical properties disappear in the apical ectoderm, the presumptive material of the distal phalanx is still enclosed in a uniform mass of undifferentiated mesoderm.

If it is assumed that these changes are the expression of a sudden breaking up of the ectoderm-mesoderm relationships into five limited areas of the marginal border, it must also be assumed that the early mesodermal columns are the only parts of the foot-plate mesoderm that have retained the ability to maintain the apical ectoderm in good functional condition.

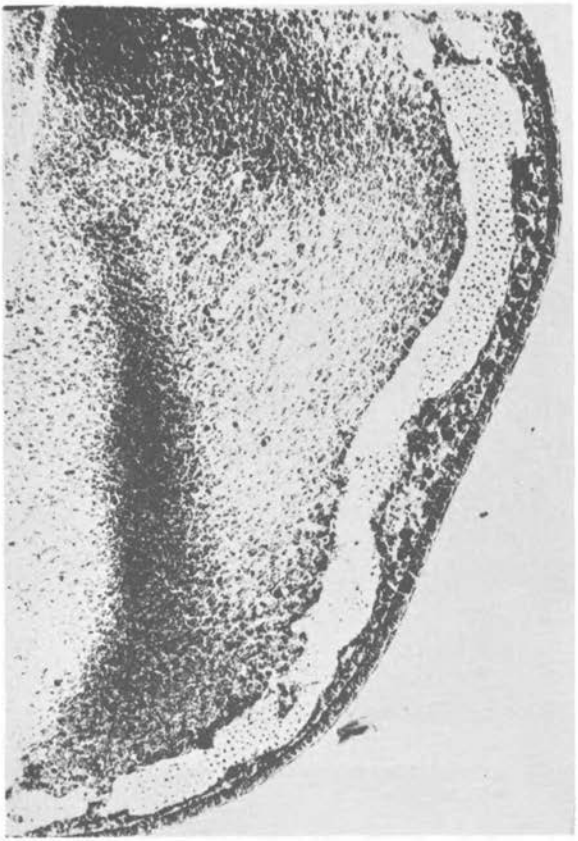

FIGURE 23 Mouse embryo at 12 days: postaxial part of a frontal section through the hindlimb bud (Unna-Brachet staining). Many dying cells are shown in the outer mesoderm lying in the fourth interdigital area, between the ectodermal ridge and the marginal venous sinus.

Although such a redistribution of the mesodermal maintenance factor has not yet received experimental confirmation in the chick embryo, the hypothesis can be supported by a particular histochemical observation in the mouse hindlimb bud. In this species, the early petaradiating blastemata differ from all other preskeletal rudiments by their selective ATP-phosphohydrolase activity (Figure 26). This reaction initially is distributed in all the mesodermal columns of the 12-day limb bud. It further decreases and later disappears in a proximodistal direction as soon as the proximal material starts chondrifying (Figure 27). It is therefore interesting to consider this particular enzymatic activity as a metabolic

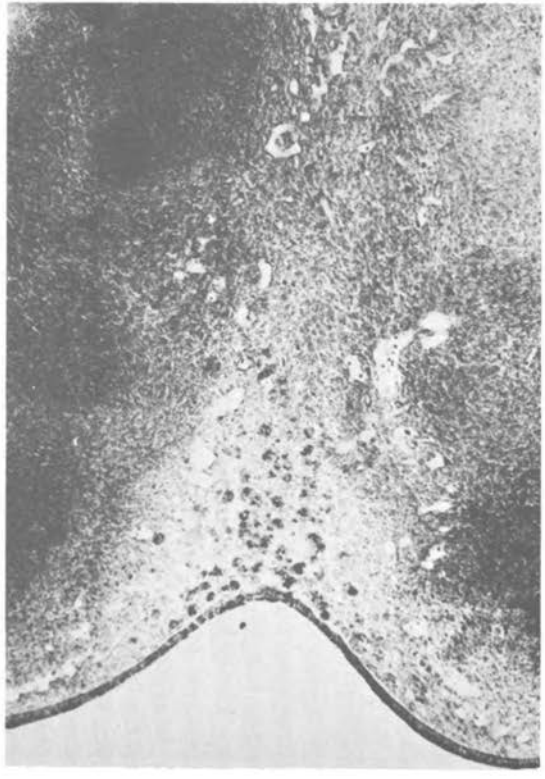

FIGURE 24 Mouse embryo at 13 days: detail of a frontal section through the isolated hindlimb bud (Unna-Brachet staining). Many dying cells in the third interdigital area of the mesoderm are seen.

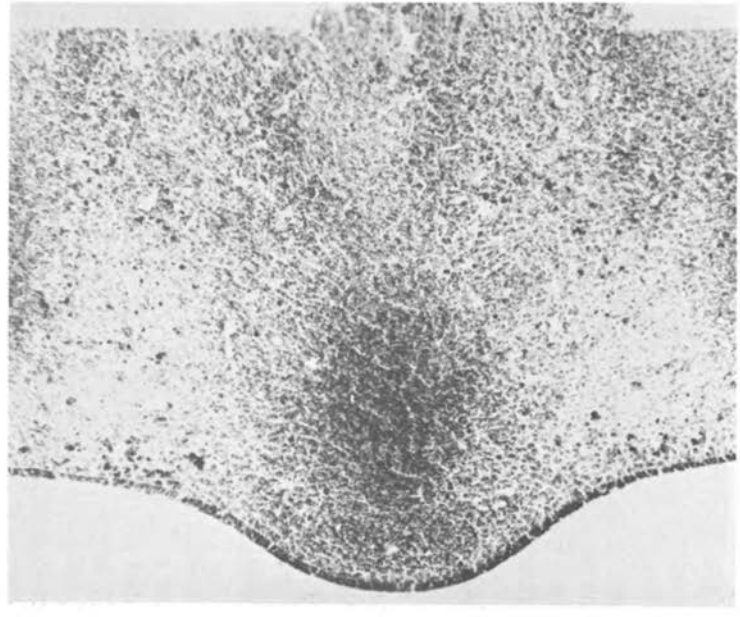

FIGURE 25 Mouse embryo at 13 days: detail of a frontal section through the isolated hindlimb bud (Unna-Brachet staining). Large amounts of RNA are present in the apical ectoderm of the third digital bud. Numerous dying cells can be seen in the superficial mesoderm of the two neighboring interdigital areas.

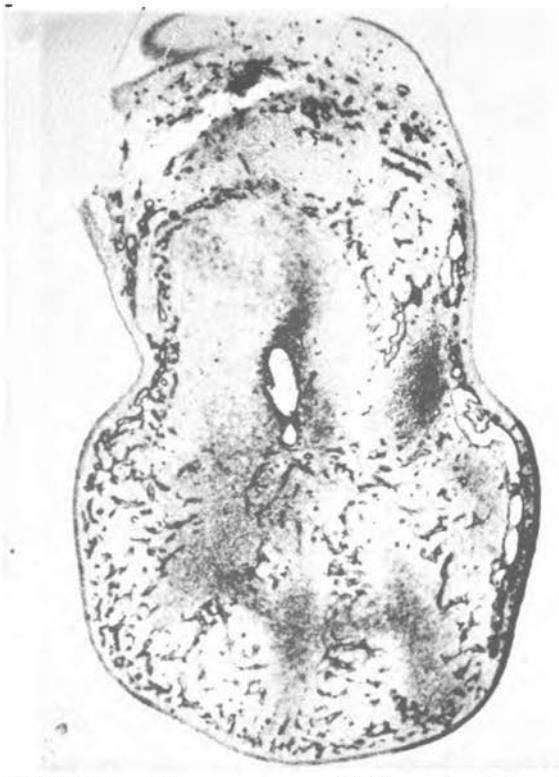

FIGURE 26 Mouse embryo at 12 days: frontal sections of the isolated right hindlimb buds, activity of the ATP-phosphohydrolase. Selective mesodermal activity can be shown in the entire radiating blastemata of the foot plate.

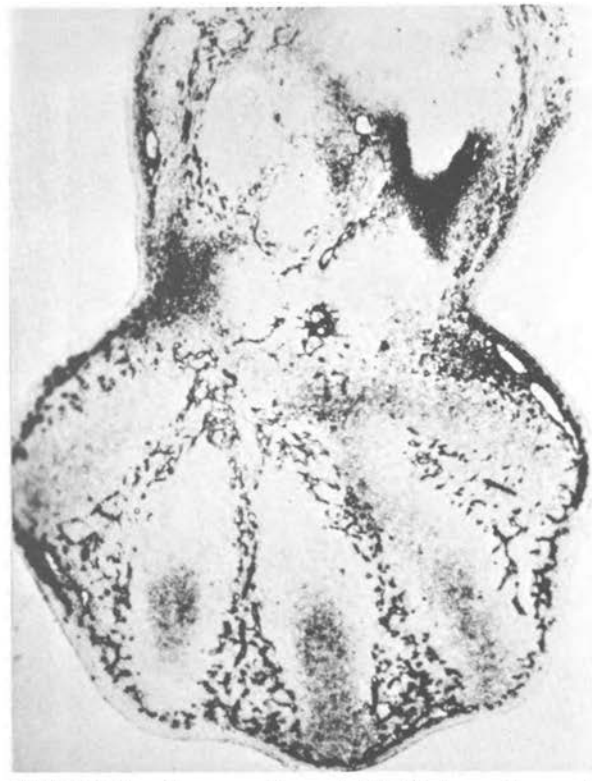

FIGURE 27 Mouse embryo at 12-1/2 days: Same activity has disappeared in the proximal parts of these blastemata, which remain positive in their undifferentiated distal two thirds.

expression of the morphogenetic properties presumably endowed by the early mesodermal blastemata.

At later stages, the final individuation of digits involves a late and probably passive invagination of the ectoderm into the necrotic areas of the interdigital mesoderm.

4. Histochemical Changes Involved in the Early Differentiation of Mesoderm into Cartilage, Articular Tissue, Muscle, and Tendon

Various histochemical changes occur during the differentiation of mesoderm into skeletal, muscular, or tendinous tissue. Some of these later aspects of limb morphogenesis have been studied comparatively in the mouse and mole embryos of corresponding stages (Milaire, 1963). They may be summarized briefly as follows:

a. Formation of Cartilage and Perichondrium. In both species and in all segmental territories, the preskeletal rudiments appear as compact and very basophilic blastemata that can hardly be distinguished from the surrounding mesoderm (Figure 21). It has already been shown that this initial organization starts in the proximal territories and gradually proceeds in a proximodistal direction. The femoral or humeral blastema is continued without any segmental demarcation by the twinned elements of the zeugopodium, which are first incorporated into a common plate of compact mesoderm. Cell degeneration occurs later in the axial part of this plate and divides it into a preaxial and a postaxial blastema. Each of these joins distally with a common mass enclosing the presumptive material of the proximal basipodium; the latter is further continued by the five radiated columns, including the material for the distal basipodium, the metapodium, and the digits.

During a long period, the whole preskeletal model remains uniformly composed of compact and basophilic mesodermal cells, and no distinction can be made between the presumptive articular areas and the precartilaginous material itself. At an advanced stage that corresponds approximately to the appearance of the third mesodermal column in the foot plate, chondrification starts in the humeral or femoral blastema, involving selective histochemical changes.

In the mole, a slight deposit of glycogen appears in the young chondroblasts; this is followed a few hours later by the appearance of a thin exocellular sheet of periodic acid-Schiff (PAS) positive and salivary resistant material (Figure 28). At later stages, more

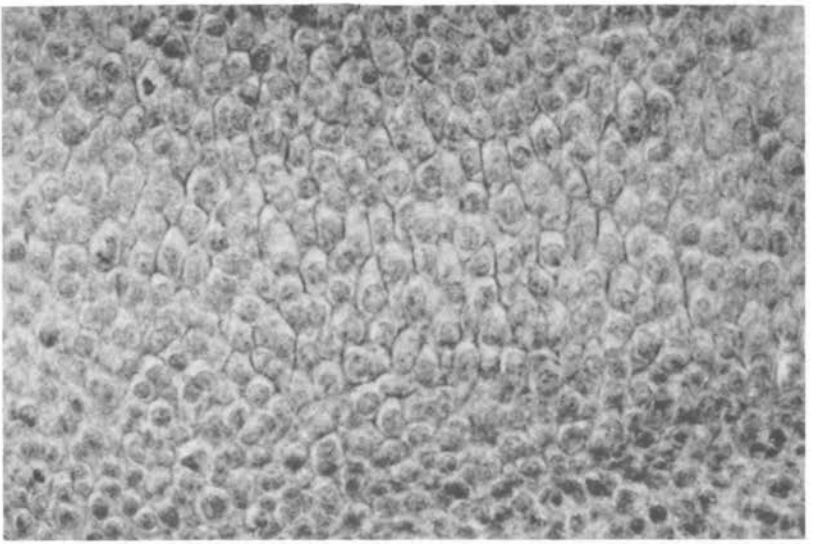

FIGURE 28 Radius precartilage of the mole embryo, PAS reaction after salivary digestion. A thin sheet of mucopolysaccharide is demonstrated between the precartilaginous cells.

and more glycogen is found in the precartilaginous cells, while the amount of exocellular ground substance increases considerably. In the mouse, the chondroblasts remain devoid of glycogen, but the stage of chondrification can be appreciated by observing the increasing thickness of the PAS positive ground substance.

In both species, very selective enzymatic properties appear in the early perichondrial cells as soon as the more central elements start chondrifying. Among the various dephosphorylating activities demonstrated in this material, that of alkaline phosphatase appears at the earliest stage, even before any exocellular mucopolysaccharide can be detected in the inner precartilaginous mass. At later developmental stages of the mouse limb buds, the same perichondrial cells acquire acid phosphatase, ATP- and AMP-phosphohydrolases (Figure 29). Such reactions have not yet been observed in the limb buds of the mole.

Wherever the changes mentioned above take place, in the precartilaginous cells or in the surrounding perichondrial cells, they progress from one skeletal piece to the next in a proximodistal direction; moreover, when they reach the carpometacarpal (or tarsometatarsal) level, successive steps of their progression can be demonstrated in one single skeletal element (Figure 30).

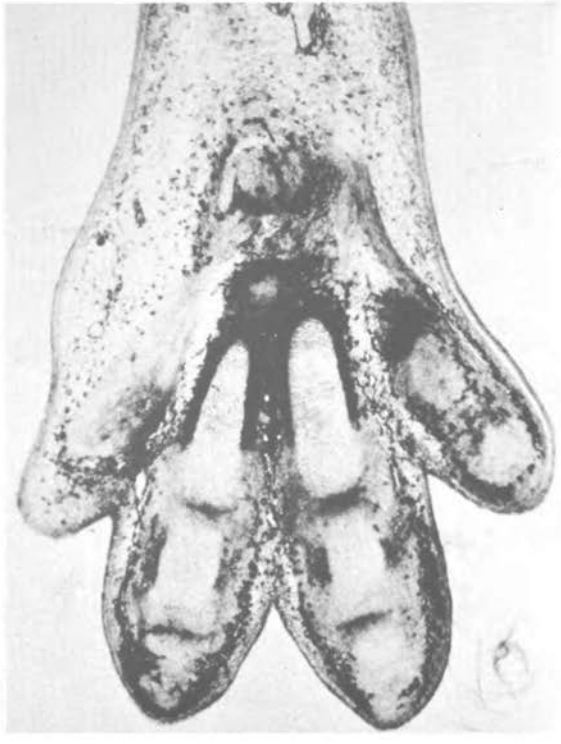

FIGURE 29 Mouse hindlimb bud at 15 days: frontal section, activity of ATP-phosphohydrolase. Strong enzymatic reaction is present in the precartilaginous tissue of the metatarsus and in the prearticular interzones.

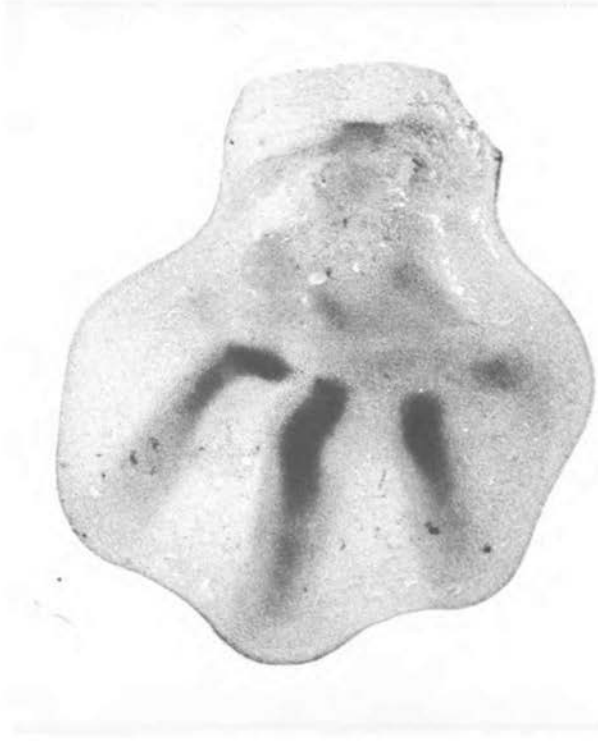

FIGURE 30 Frontal section of a mole forelimb bud, PAS reaction. Glycogen is present in the proximal part of the three middle radiating precartilages of the hand. Concentration of the glycogen decreases in a proximodistal direction.

Such observations thus provide delayed evidence of the earlier proximodistal determination of the limb-bud mesoderm as it was established in the chick limb buds by carbon marking experiments. There are, however, two exceptions which show that the chronological correlations between the metabolic changes involved in skeletal differentiation and the earlier organization of the undifferentiated mesoderm can vary from one region to the other. The first exception concerns the proximal elements of the carpus, which start chondrifying long after the distal ones, even later than some of the metacarpal rudiments. The second exception concerns the metacarpals (and metatarsals) themselves, where chondrification does not proceed according to the same caudocephalic gradient that had initially characterized the early organization of the footplate mesoderm. It starts simultaneously in the second, third, and fourth metacarpals before reaching the fifth and, still later, the first.

b. Histochemical Properties of the Early Prearticular Interzones. All presumptive articular regions are initially incorporated in the common mesodermal model of the skeletal structures. At early stages, the mesoderm present in the prearticular interzones offers the same appearance as the neighboring precartilaginous material. It is thus composed of a compact mesoderm highly charged with RNA. As soon as the precartilaginous material starts chondrifying, the RNA content decreases in the contiguous prearticular mesoderm. At the same time, a very selective acid phosphatase activity becomes demonstrable in the limited region that later will be replaced by the articular cavity (Figure 31). The reaction characterizes very small intracytoplasmic granules, probably lysosomes, which differ considerably from the large positive inclusions usually found in the dying cells. In fact, not a single degenerating cell can be detected in these regions in sections stained by the Unna-Brachet. On the other hand, no particular histochemical change occurs in the mesoderm surrounding the positive areas, a material that later will give rise to the articular capsule and ligaments. At later stages, the reaction of acid phosphatase increases in the interzone, where, in addition, strong ATP- and AMP-phosphohydrolase activities become demonstrable (Figure 32).

At the latest developmental stages studied so far, the articular cavity has not yet been formed, even in the more advanced proximal regions of the limb. However, more detailed studies pursued on human embryos (Andersen and Bro-Rasmussen, 1961, and more recently Andersen, 1963, 1964) have shown that most of the

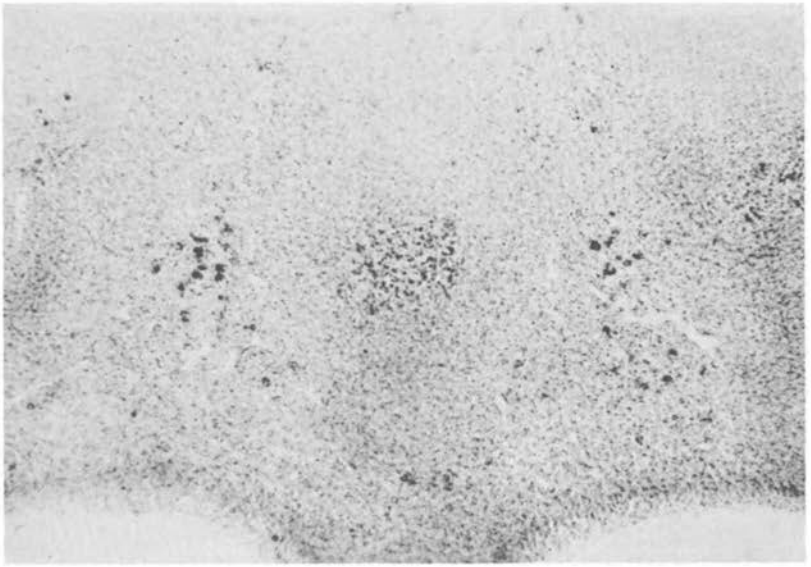

FIGURE 31 Mouse embryo at 13 days: detail of a frontal section through the hindlimb bud, acid phosphatase reaction. Reaction is positive in the prearticular interzone of the future metatarsophalangeal joint; it is also very strong in the dying cells present in the adjacent interdigital mesoderm.

mesoderm present in the articular interzone undergoes a late cartilaginous transformation and finally is incorporated into the epiphyseal cartilage of the jointed skeletal pieces. It may therefore be assumed that the selective histochemical changes observed in the corresponding regions of mouse embryos are related to a temporary inhibition of chondrogenesis; the study of later stages probably will clarify this problem.

c. Formation of Muscles and Tendons. The formation of soft tissues, and more particularly that of the musculotendinous rudiments, begins considerably later than that of the skeletal structures. Therefore the stages studied hitherto from the histochemical point of view concern only the initial changes involved in this matter, that is, the early individuation of the premuscular mesoderm and its further organization into identifiable morphological units.

During a long period, the presumptive material of muscles and tendons remains included in the outer mantle of undifferentiated mesoderm, surrounding the central core of preskeletal tissue. A thin layer of loose mesoderm is interposed between these areas;

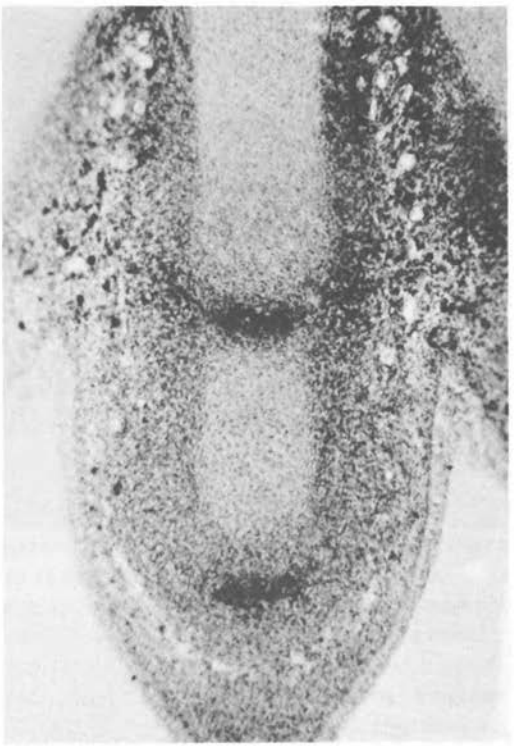

FIGURE 32 Mouse hindlimb bud at 15 days: frontal section through the third digit, acid phosphatase reaction. Strong reaction in the prearticular interzones of the mesoderm.

it provides a path for the largest blood vessels. As soon as the outer mesoderm can be distinguished from the inner preskeletal material, it appears to be much more abundant ventrally than dorsally (Figure 33). This dorsoventral asymmetry can be observed in any transverse section perpendicular to the limb-bud axis, and it is thus present in each segmental territory. It is interesting to note that during the same developmental period, important structural as well as histochemical differences can be observed between the dorsal and ventral ectoderm. Over the whole ventral aspect of the limb bud, the ectoderm remains thick, with a high concentration of RNA, and demonstrates a high level of alkaline phosphatase activity (Figure 34). Conversely, the dorsal ectoderm is thin, contains

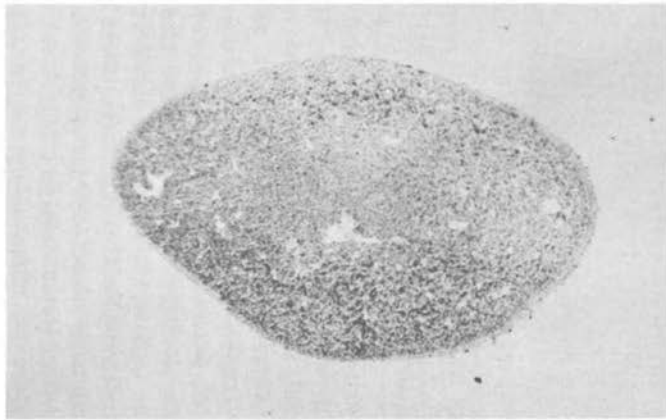

FIGURE 33 Mouse embryo at 11-1/2 days: transverse section perpendicular to the proximodistal axis of the hindlimb bud, at the level of the zeugopode (Unna-Brachet staining). Amount of outer basophilic mesoderm is higher ventrally than dorsally.

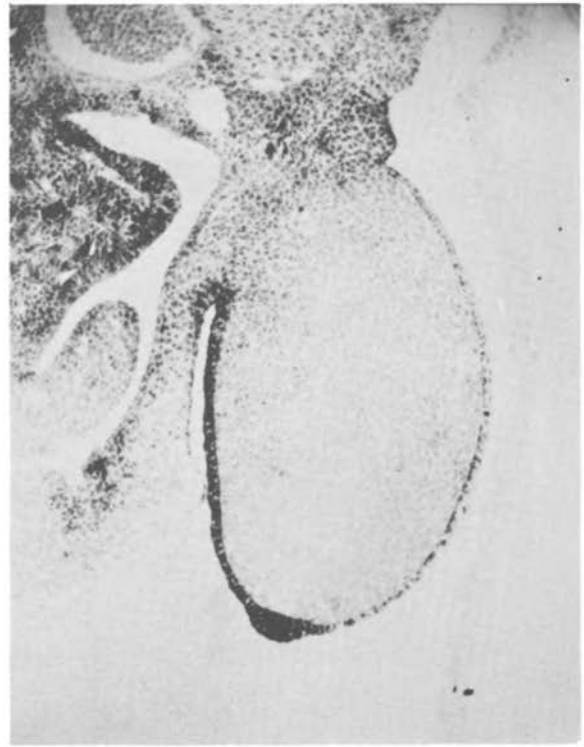

FIGURE 34 Mouse embryo at 10-1/2 days: transverse section of the forelimb bud in situ, alkaline phosphatase reaction. Strong enzymatic activity in the ventral ectoderm and in the apical ectodermal ridge.

little RNA, and shows a very low level of alkaline phosphatase. The observations have led us to assume that the initial amount of premuscular mesoderm might be controlled locally by ectodermal influences. Such an assumption requires experimental confirmation. At present the assumption is based upon suggestive events occurring correlatively in the mesoderm and in the overlying ectoderm and upon the existence of histochemical similarities between the ventral ectoderm and the apical ectodermal ridge.

After a long growing period during which the outer mesoderm retains a uniform and undifferentiated appearance, this material suddenly loses its relationships with the overlying ectoderm and comes to lie close to the deep precartilaginous rudiments. This modification occurs differently in the proximal segments from the way it does in the more distal foot plate. Priximally, it involves a local transformation of the most superficial cells into loose mesenchyme. In the foot-plate area, a similar transformation occurs but is accompanied by an active migration of the deeper cells toward the underlying precartilaginous rudiments. Such changes start in the more proximal territories, before the penetration of nerve fibers into the limb bud; they later proceed gradually in a proximodistal direction.

At later stages, various successive cleavages occur in the resulting masses of mesoderm, giving rise to several groups of premuscular and pretendinous blastemata. In each segment of the limb, the number of premuscular rudiments is higher ventrally than dorsally. This final predominance of the flexor musculature over the extensors is probably related to the earlier dorsoventral asymmetry demonstrated in the ectoderm.

Our observation also shows clearly that each part of a muscle is formed by the local transformation of mesoderm initially laid down in the corresponding region of the limb bud. For example, there is no doubt that the presumptive mesoderm of the tendinous portion of the flexor digitorum is present initially in the foot-plate area, while the mesoderm of the corresponding muscular portion has originated in the more proximal zeugopodium.

Finally, apart from their high content of RNA, the early myoblasts do not show any other histochemical peculiarity during the entire period of musculotendinous organization. The reason for this, in all probability, is that the differentiation of myoblasts into muscular fibers occurs long after the organization of the presumptive cells into morphological units.

Observations on Abnormal Limb Buds

In the literature, many examples can be found to demonstrate how a detailed comparative analysis of normal and mutant embryos can improve our understanding of congenital malformations in mammals. By retracing the successive developmental steps of the affected structure, it has been possible to translate a complex anatomical situation to a limited disorder occurring at a particular developmental stage. Grüneberg's expression, "pedigree of causes," has become classical.

If the morphological changes occurring in the developing rudiments are studied parallel with the histochemical changes, a similar comparative analysis acquires double importance. First, this study can approach more closely the primary genic action by revealing some early metabolic disorder preceding any morphological modification. Secondly, the histochemical changes observed in mutant embryos may sometimes improve understanding of the histochemical properties demonstrated in the corresponding regions of normal embryos.

This statement is based upon observations collected in the developing hindlimb buds of three different strains of mutant mice. In each of the strains, the skeleton of the foot is affected differently by the genetic disorder.

1. Oligosyndactylism

Oligosyndactylism, as described by Grüneberg in 1956, is a dominant congenital malformation leading to various degrees of fusion between the second and third digits in all four limbs. The posterior limbs are universally more severely affected than the anterior limbs, the involvement of skeletal structures being usual in the posterior limbs and occasional in the anterior. Eventual bony fusions generally start at the basal phalanges and thence spread distally. In severe cases, secondary fusion may occur between the second and third metatarsals, which, however, are always laid down as separate precartilages. From later developmental analysis, it was concluded that the final anatomical abnormalities were attributable to a reduction of the amount of mesoderm in the preaxial part of the foot plate (Grüneberg, 1961). This reduction becomes demonstrable in the forelimb buds at the 11-day stage and occurs a few hours later in the hindlimb buds. No other morphological change was found in the mesoderm or in the ectoderm. According

to the amount of mesoderm available, the second and the third digital rays condense more or less close to each other or even appear as a common blastema.

Histochemical study of the affected limb buds has confirmed that the first detectable injury occurs in the preaxial mesoderm. It also has shown that the corresponding portion of the ectodermal ridge undergoes an apparently normal evolution. However, as shown in Figure 35, it was possible to trace the first genic lesion much further back histochemically than is possible with standard morphological techniques. Degenerating cells were demonstrated in the preaxial mesoderm of 10-1/2-day forelimb buds, and similar necrotic changes appear in the corresponding region of the hindlimb buds as soon as the embryo reaches the stage of 10 days and 16 hours. At these stages, the foot plate still has a normal shape. Later, the number of affected cells increases rapidly and is still high when the resulting reduction of the amount of mesoderm causes a preaxial flattening of the foot-plate margin. We know that cell degeneration occurs in the 12-day normal limb buds around the distal extremity of the first radiated blastema. This normal event takes place in the deep mesoderm facing the preaxial edge of the apical ridge, that is, in the same region which, in the mutant limb buds, was affected earlier by similar necrotic changes. In the 12-day Os/+ limb buds, many residual dying cells affected by the genic injury are still present. They contribute to the enlargement of the

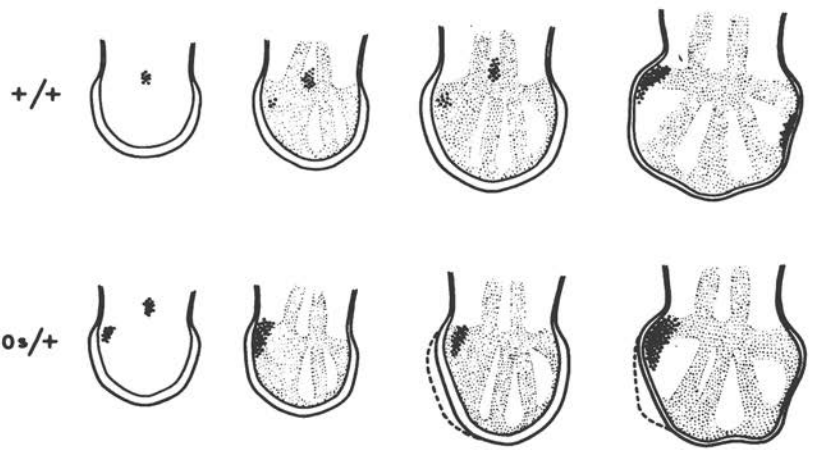

FIGURE 35 Distribution of the necrotic cells in the preaxial mesoderm of the normal (+/+) and the oligosyndactylous (Os/+) mouse hindlimb bud.

preaxial necrotic area with respect to that of the normal limb buds. The presence of acid phosphatase in the affected region is shown in Figures 36 and 37 at two successive developmental stages.

Thus it may be concluded that the reduction of preaxial mesoderm responsible for oligosyndactylism is the result of a regional degeneration occurring at the earliest stages of the foot-plate formation.

Despite the apparent maintenance of normal properties in the ectoderm, it is highly probable that the genetic injury modifies in some manner the morphogenetic interactions between the affected mesodermal area and the overlying portion of the ectodermal ridge. It is hoped that further histochemical studies will clarify this problem.

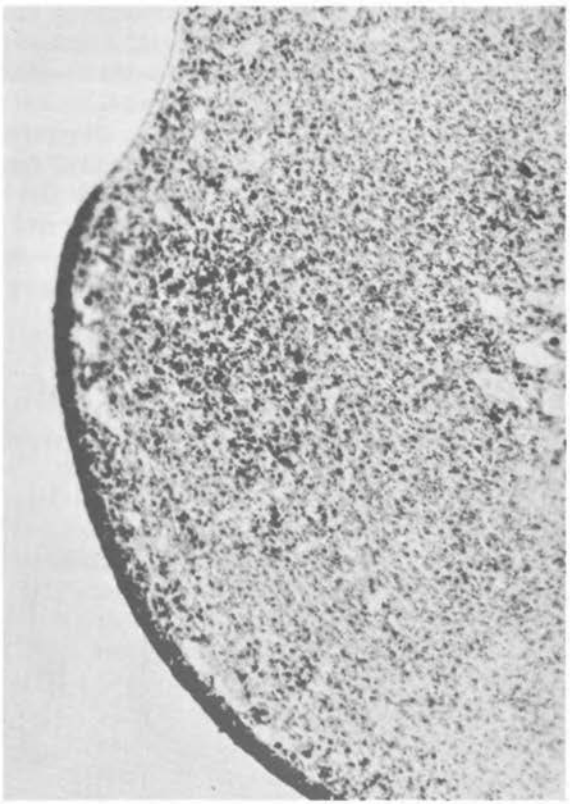

FIGURE 36 Strong acid phosphatase reaction in the dying cells present in the preaxial mesoderm of Os/+ mouse hindlimb bud at stage 10-1/2 days.

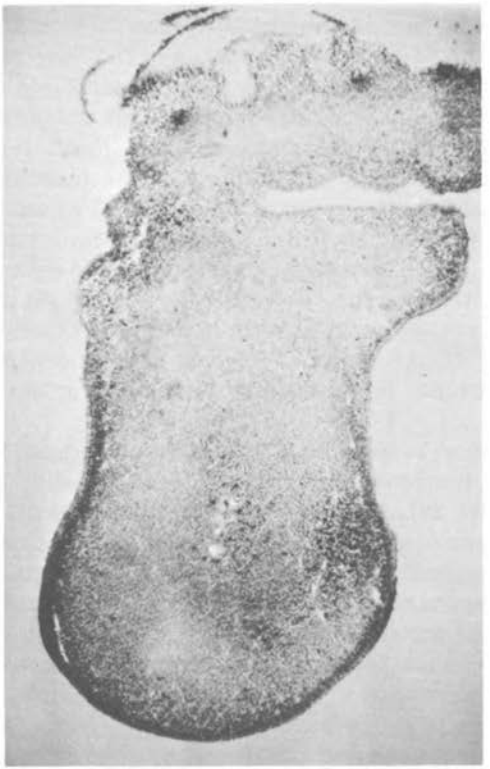

FIGURE 37 Strong acid phosphatase reaction in the dying cells present in the preaxial mesoderm of Os/+ mouse hindlimb bud at stage 11 days.

2. Syndactylism

The anatomical lesions produced by the recessive gene for syndactylism were described by Grüneberg in 1956. Various types of fusions occur between the third and fourth digits, the second digit occasionally being involved. As in oligosyndactylism, digits of the forelimbs usually are joined by soft tissues only, and those of the hindlimbs by bony tissues. Here, however, the fusions proceed from the distal to the more proximal phalanges, and the metatarsals are never affected. In mild cases, the distal phalanges of two adjacent digits form a single skeletal unit, while the proximal ones rest close to each other in a common cutaneous sheath. In more severely affected limbs, all three phalanges of two or three neighboring digits may be coalescent. In a later morphological analysis

of the developing mutant limb buds (Grüneberg, 1960), it was discovered that the earliest manifestation of the 'sm' gene appeared at the 10-1/2-day stage as a significant hyperplasia of the apical ectodermal ridge and other parts of the limb epidermis. At later stages, a very peculiar deformation occurs that forces the foot plate to bend in a plantar direction. As a mechanical consequence of this deformation, the radiated mesodermal columns of the middle digits crowd together at their distal extremity and fuse to variable degrees. Dr. Grüneberg suggests that the enlargement of the foot plate and the resulting deformity are due to an increased stimulation of the mesodermal growth by the hyperplastic ectodermal ridge. The skeletal fusions, therefore, may be considered a secondary consequence of a systemic tendency to epidermal hyperplasia.

Histochemical results from his own studies have led the writer to understand the genesis of syndactylism in a slightly different manner (Figure 38). Frontal sections of the isolated limb buds have first shown that the hyperplastic transformation of the ectodermal ridge affects the preaxial half of this structure only. This modification appears at the early 10-day stage. The abnormal thickness of the ectoderm is maximal at the 11-day stage, gradually decreasing up to the 13th day. It is important to note that the hyperplastic preaxial portion of the apical ridge contains twice as many degenerating cells as the normal structure. This evidence strongly suggests that the increased cell proliferation occurring in the ectoderm is not the result of an increased mesodermal stimulation but a straight genetic disorder acting directly on the ectoderm.

At later stages, no structural or histochemical evidence was found of mesodermal overgrowth. RNA and mitotic figures were found normally distributed in the mesodermal field. On the other hand, peculiar deformations of the ectodermal cells in the vicinity of both extremities of the apical ridge indicate that the rigidity of the modified apical ectoderm impedes the normal expansion of the footplate mesoderm. It appears as if the mesoderm continues to grow normally in an inextensible ectodermal sac. The resulting transitory deformation of the foot plate then forces the three middle radiated blastemata to converge distally. The ectodermal ridge finally reaches its maximal thickness when the fourth blastema begins condensing. This provides a satisfactory explanation of the constant involvement of the fourth digit in the process of fusion.

The changes occurring in the more distal areas of the foot plate strongly indicate that the final coalescence of digits is the result of an abnormal distribution of the morphogenetic factors that were

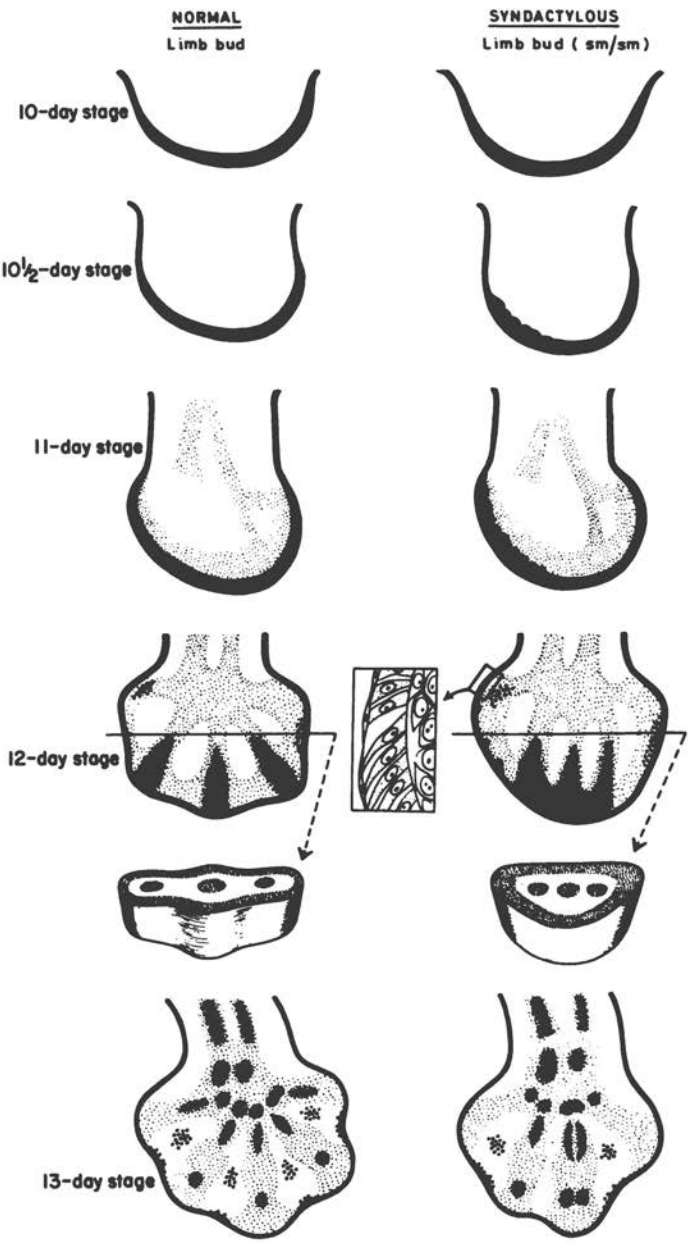

FIGURE 38 Schematic aspects of the normal and the syndactylous (sm/sm) hindlimb bud of the mouse embryo, from the 10th to the 13th day. (See text.)

postulated in normal rudiments. The ATP-phosphohydrolase activity that normally characterizes the digital portion of each radiated blastema was found uniformly distributed in the marginal mesoderm of syndactylous limb buds (Figure 39). In addition, a high RNA content is maintained in the overlying portion of the ectodermal ridge (Figure 40), as if the respective maintenance influences exerted by the deformed mesodermal rays had united distally and had therefore attained a large portion of the marginal ectoderm. Consequently, the whole mesoderm lying distally with respect to the deformed metatarsal rays undergoes a digital transformation, without any interdigital involution.

In conclusion, the genesis of syndactylism involves three suc-

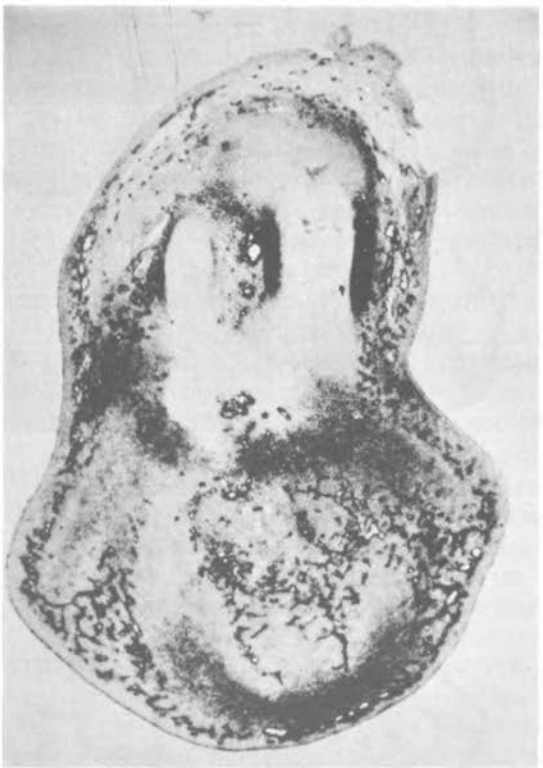

FIGURE 39 Syndactylous (sm/sm) mouse embryo at 12 days: frontal section of the isolated hindlimb bud, activity of the ATP-phosphohydrolase. Strong reaction is demonstrated in the three middle radiated blastemata of the foot plate (partially visible on the section) and in the marginal layer of mesoderm in which they converge distally.

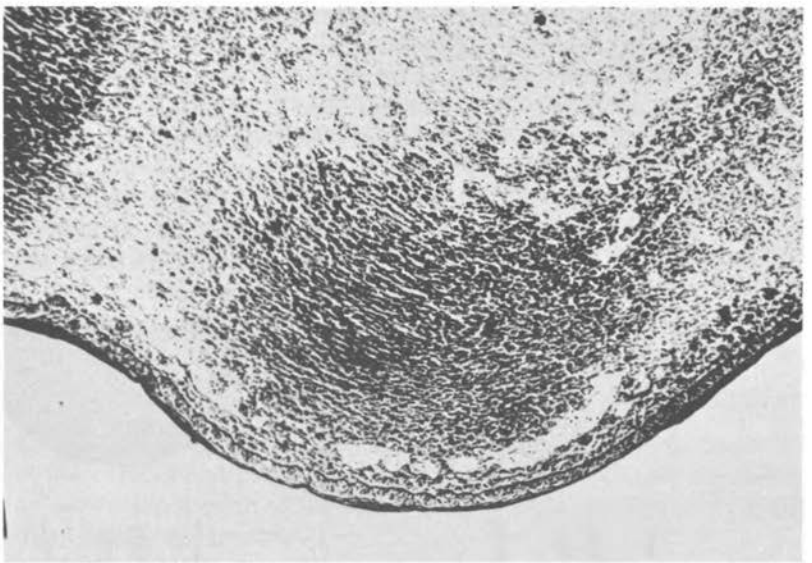

FIGURE 40 Sm/sm hindlimb bud of the mouse at 13 days: frontal section of the third digital rudiment fused with the fourth. Superficial residues of the hyperplastic lesion are still present in the apical ectoderm, the basal cells of which show a high RNA content.

cessive and different kinds of modifications. The early genic injury is a transitory hyperplasia of the preaxial ectodermal ridge. The resulting rigidity of the apical ectoderm provokes a mechanical deviation of the early metatarsal rudiments. This deformation then modifies the distribution of the mesodermal maintenance factor in such a way that presumptive interdigital mesoderm is used in the formation of digital structures.

3. Dominant hemimelia

Besides a constant absence of the spleen, heterozygous animals for dominant hemimelia show polymorphous skeletal defects in all four limbs (Searle, 1959). One of the most frequent abnormalities is the presence of an additional phalanx in the first toe. This limited aspect of the malformation will be considered in the developing hindlimb bud.

Among the various modifications that can be observed in the hindlimb buds of different 10-day mutant embryos, one might be

considered the primary injury leading to hyperphalangia (Figure 41). It consists in a uniform distribution of the basophilic mesoderm on both sides of the limb-bud axis. We know that in normal limb buds this material is predominant postaxially. Of course, it still is impossible to know which of such affected limb buds would have formed a hyperphalangic first toe.

The first obvious indication of this particular abnormality appears at the 11-day stage as a slight overgrowth in the extreme distal portion of the footplate. At this time it is observed that the

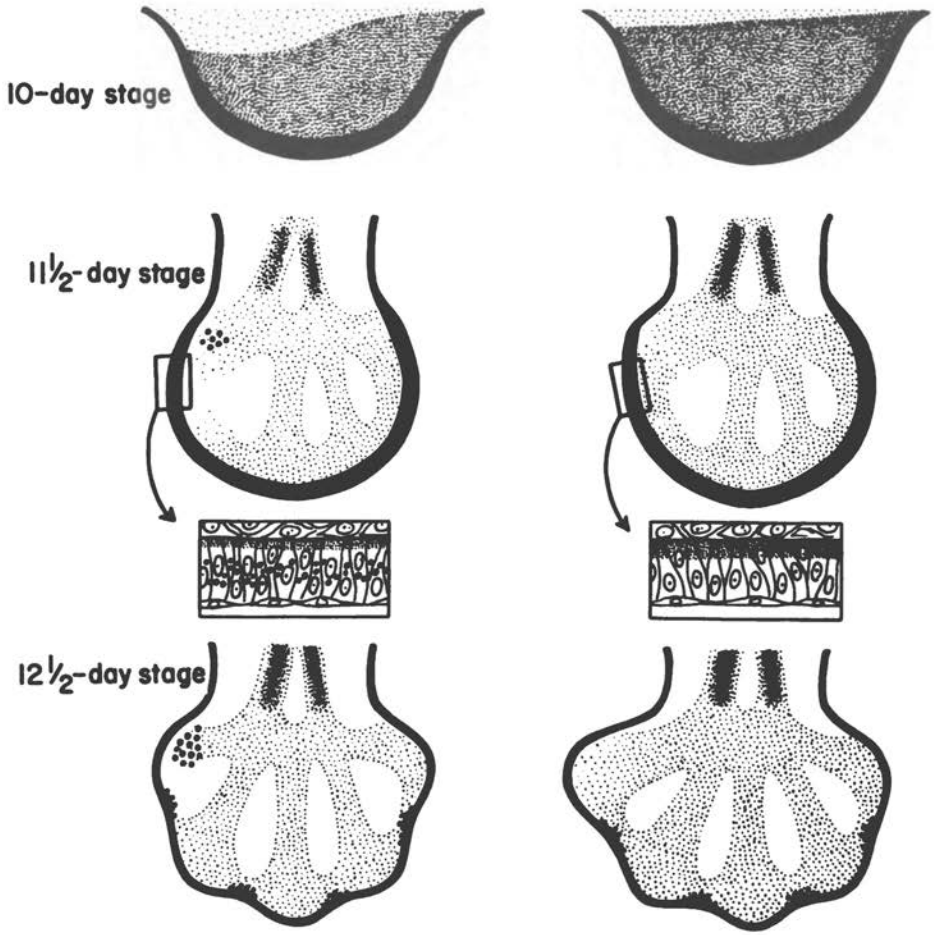

FIGURE 41 The genesis of hyperphalangia of the first toe in Dh/+ limb buds.

preaxial mesoderm, as well as the corresponding part of the ectodermal ridge, is completely free of any degenerating cells. Furthermore, this part of the marginal ectoderm contains much more RNA than normal limb buds. In normal hindlimb buds of the same stage, several dying cells are already present in the preaxial mesoderm, and the preaxial half of the ridge is entirely pycnotic. At later stages, the first radiated mesodermal column of the abnormal rudiments is excessively long, and the overlying ectoderm does not show any cytolytic change.

These observations strongly suggest that hyperphalangia of the first toe results from the occurrence of increased morphogenetic properties in the preaxial mesoderm. This seems to result from the excessive amount of basophilic mesoderm present in the preaxial half of the bud, which is responsible for the maintenance of better structural and functional conditions in the overlying portion of the ectodermal ridge. Consequently, the ectodermal ridge induces an excessive growth of the underlying mesoderm and the formation of an additional phalanx.

If the above supposition is correct, the modifications involved in the genesis of hyperphalangia appear to be the reverse of those leading to oligosyndactylism. However, the apical ectoderm did not show any sign of an increased degeneration.

Finally, it may be pointed out that the observations made in Dh/+ limb buds strongly suggest that the cytolytic changes occurring normally in the preaxial mesoderm affect a material that would have formed a third phalanx in the first toe.

Conclusions

On the basis of our histochemical investigations, the following series of general conclusions may be formulated:

1. In mammalian as well as in chick embryos, the descriptive study of the developing limb buds provides structural and histochemical evidence of uninterrupted morphogenetic interactions between the undifferentiated mesoderm and the overlying ectoderm.

2. In all species considered, the initial ectodermal activities are uniformly distributed over the entire ventral surface of the bud. They later become predominant in a more limited ventro-apical region that soon transforms into an apical ectodermal ridge. This ridge forms earlier in chick than in mammalian embryos. In mammals, the role of the ectodermal ridge seems to be selectively associated with the genesis of the distal limb segment. The

only demonstrable effect of the ectodermal influence is a regional stimulation and consequently a topographical control of the growing activities in the underlying mesoderm.

3. Different kinds of mesodermal properties, including enzymatic activities, were found asymmetrically distributed on both sides of the limb-bud axis. These observations may be considered histochemical evidence of the mesodermal maintenance factor discovered in the chick embryo by experimental methods.

4. The changes involved in the formation of digits in mammals are strongly suggestive of a late redistribution of the ectoderm-mesoderm relationships in the distal part of the footplate.

5. The developmental modifications observed in three different kinds of limb abnormalities have provided further evidence of a reciprocal dependence of the mesoderm and the apical ectoderm in the genesis of the distal limb segment.

REFERENCES

Andersen, H., "Histochemistry and Development of the Human Shoulder and Acromioclavicular Joints With Particular Reference to the Early Development of the Clavicle," Acta Anat., 55, 124-165 (1963).

Andersen, H., "Development, Morphology and Histochemistry of the Early Synovial Tissue in Human Foetuses," Acta Anat., 58, 90-115 (1964).

Andersen, H., and F. Bro-Rasmussen, "Histochemical Studies on the Histogenesis of the Joints in Human Fetuses, With Special Reference to the Development of the Joint Cavities in the Hand and Foot," Amer. J. Anat., 108, 111-122 (1961).

Chaube, S., "On Axiation and Symmetry in Transplanted Wing of the Chick," J. Exp. Zool., 140, 29-77 (1959).

Detwiler, S. R., "On the Time of Determination of the Anteroposterior Axis of the Forelimb in Ambystoma," J. Exp. Zool., 64, 405-414 (1933).

Grüneberg, Hans, "Genetical Studies on the Skeleton of the Mouse XVIII. Three Genes for Syndactylism," J. Genet., 54, 113-145 (1956).

Grüneberg, Hans, "Genetical Studies on the Skeleton of the Mouse XXV. The Development of Syndactylism," Genet. Res., 1, 196-213 (1960).

Grüneberg, Hans, "Genetical Studies on the Skeleton of the Mouse XXVII. The Development of Oligosyndactylism," Genet. Res., 2, 33-42 (1961).

Hertwig, O., Handbuch der vergleichenden und experimentellen Entwicketungslehre der Wirbeltiere, Fischer, Jena, pp. 166-338 (1906).

Kieny, M., "Rôle Inducteur du mésoderme dans la différenciation précoce du bourgeon de membre chez l'embryon de Poulet," J. Embryol. Exp. Morph., 8, 457-467 (1960).

Milaire, J., "Contribution à l'étude morphologique et cytochimique des bourgeons de membres chez le Rat," Arch. Biol., 67, 297-391 (1956).

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

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

Milaire, J., "Etude morphogénétique de trois malformations congénitales de l'autopode chez la Souris (syndactylisme - Brachypodis-me - Hémimélie dominante) par des méthodes cytochimiques," Acad. Roy. Med. Belg., Mem., 16, 1-120 (1965).

Milaire, J., "Etude histochimique des premiers stades du développement des membres chez le Poulet," C. R. Assoc. Anat., 51é Réunion, Marseilles (in press).

Saunders, J. W., and Gasseling, M. T., "Trans-Filter Propagation of Apical Ectoderm Maintenance Factor in the Chick Embryo Wing Bud," Develop. Biol., 7, 64-78 (1963).

Searle, A. G., "Hereditary Absence of Spleen in the Mouse," Nature, 184, 1419 (1959).

Environmental Factors in Human Teratology

SIDNEY Q. COHLAN, M.D.
Professor of Clinical Pediatrics
New York University Medical Center
New York, New York

This presentation will offer a slight change of pace from those preceding it. Whereas the previous lecturers were concerned only with limbs, the writer will deal with the whole individual. As you all know, the thalidomide catastrophe which occurred in 1961 stimulated a great deal of interest in malformations. To a large extent this interest may be responsible for our presence here. Since 1961 a mountain of literature has developed on the effects of drugs that will—or may—produce damage to the fetus if taken during pregnancy. There has been much writing, theorizing, hypothecating, and warning, but few new clinical facts.

In the laboratory it has been well established that a large variety of chemicals and drugs, if properly administered to the proper animal at the proper time, with patience, will produce congenital malformations. Frazier has stated that any material, given at the right time in the right way to the right animal, can produce a congenital malformation. The fact that this can be done has stimulated

many laboratory investigators to produce malformations with a variety of agents. These laboratory experiments no longer suffice in terms of today's needs—knowledge of the mechanisms involved. We were delighted to hear Dr. Milaire's presentation, as it is an earnest attempt to learn more about basic biochemical enzymatic mechanisms. He is a modest man, but it must be noted that one or two of the excellent staining techniques used today bear his name.

A brief review of what we know about the etiology of human malformations is in order. About 85 percent of all known clinical congenital malformations have no precise etiology. About 12 percent can be traced to known hereditary factors (genetic disturbances) or chromosomal abnormalities. About one or two percent are probably the result of environmental insults. The rest, at present, can only be categorized as "due to a subtle interaction between environmental factors and the genetic milieu."

In 1942 Courier and Yost, while experimenting with rabbits, found that the female fetus could be masculinized by the use of ethynyl testosterone. It was not until 1960 that Wilkins was able to collect about 70 cases of newborn infants whose female genitalia had been masculinized by the administration to the mother of various progestational compounds that have androgenic effects. The female infants were born with large clitorises and various stages of labial fusion. Androgens (testosterone preparations) when given before the 12th week of pregnancy will produce similar effects. There have been four or five instances where estrogens have, for some reason, produced a paradoxical effect of masculinization in the female fetus. Clinically, the risk of progestational masculinization is of a low order, because many thousands of women in this country are receiving one type or other of progestational compound for spontaneous abortion or intrapartum hemorrhage; yet the number of infants affected is relatively very small.

THE ADRENAL STEROIDS

Cortisone is a highly potent teratogen in the mouse. Cortisone given to certain strains of mice will produce a 100 percent incidence of cleft palate. In the human, the situation is not as clear. Several years ago, Bongiovanni collected a series of cases of 260 women who, during their pregnancies, received significant therapeutic doses of cortisone for appropriate indications. Two infants with cleft palate were reported. Popert reviewed another group of

75 pregnant women who received steroids for rheumatic fever, and he also found two cases of cleft palate. This incidence is higher than would be expected in the random population, but it is not so statistically significant as to preclude the use of cortisone. Where cortisone is definitely indicated, the physician should not hesitate to use it. He must be fully aware, however, that there is a potential risk to the fetus.

ANTI-CANCER AGENTS

Almost any of the cancer chemotherapeutic agents are potential teratogens. The fact that they are used in situations where they will affect cellular multiplication and division suggests that they will also affect the growing embryo. Aminopterin is one material with which we have had considerable experience. This material was investigated by Thiersch about 10 years ago in his search for an efficient abortifacient and was found to be quite effective for this purpose. About three or four milligrams per day for about 3 to 6 days will, in most instances, produce spontaneous abortion about 5 to 17 days after cessation of therapy. Unfortunately, if the fetus is not aborted, a significant percentage of the infants will be grossly deformed with defects such as hydrocephalus, spina bifida, and cleft palate. Obviously, then, aminopterin as a therapeutic abortifacient is a very dangerous teratogenic drug. Aminopterin is also a most potent teratogenic agent in the laboratory.

With many drugs there has been inadequate or inconclusive experience with respect to their role as teratogens. One hears an occasional isolated report of a pregnant woman being treated for a tumor who produced a deformed infant. However, many pregnant women treated with the same agents delivered normal babies.

TOLBUTAMIDE

This is an oral hypoglycemic agent used in the treatment of diabetes. There have been at least four reports of women treated with tolbutamide during pregnancy whose offspring were abnormal.

THALIDOMIDE

This is the most potent human teratogen known to date. Our knowledge of it is still incomplete. There is no factual evidence of the precise mechanisms involved in thalidomide embryopathy.

The next major agent in terms of environmental insult is infection. Here, too, there is a large bibliography in the literature but little precise information. There are only three diseases known to produce congenital defects. The first is rubella, about which we know more than other diseases. The second is toxoplasmosis, which is rare. The third, also rare, is salivary gland disease, sometimes called cytomegalic virus disease. The second and third of these diseases produce hydrocephalus, retinal calcification, and mental retardation. We do not understand the mechanisms involved, nor do we know why some women harboring these diseases at the right time produce normal infants and others produce abnormal infants.

In the spring of 1964 a major German measles epidemic occurred in the New York area and other parts of this country. Whereas normally in New York about 2,000 to 3,000 cases of German measles are reported annually, more than 30,000 cases were reported in 1964 (and many others went unreported). In view of this situation it was anticipated that a large number of babies would be exposed to rubella in utero.

As expected, there was a high incidence of deafness and mental retardation in the newborn. The typical rubella triad of cardiac anomalies and cataracts was also encountered. What was not expected, however, was the marked increase of a serious congenital syndrome. Many babies were born underweight and rapidly developed thrombocytopenic purpura with hemorrhage. This syndrome has not been well documented previously. Many of these children died of hemorrhage, and there was a large variety of other manifestations. These data are reported by Krugman and Cooper at New York University, where, at the time of writing, the cases of almost 200 infants who manifested this syndrome were being followed. Of the 200 infants, the laboratory was able to isolate 92 cultures of rubella virus.

This syndrome was a relatively new finding. These babies had been infected by the rubella virus in the first trimester of pregnancy, six months before they were born. At birth they were still shedding the virus despite the fact that the mother and the infant did have hemagglutinating antibody in their circulation. There were 70 babies with the purpura syndrome, from which the virus was recovered in 58. Hepatomegaly, splenomegaly, and congenital heart disease were other major manifestations. Eye lesions (cataracts, cloudy cornea, or glaucoma), adenopathy, and bone lesions were also noted. The bone lesions came as a surprise, inasmuch as they had not been reported before. The lesions were almost exclusively in the distal portions of the femur and occasionally in the proximal portion of the tibia. Areas of radiolucency, an apparent

loss of mineralization, plus a disturbance of the trabeculations at the distal end of the metaphyses of both bones, were typical findings. In the surviving infants, these lesions gradually disappeared. By the time the children were three or four months old, the bones looked normal on x rays. We do not quite understand the mechanism, as it rarely affects other bones of the skeleton. It is a reproducible effect and has been observed in various parts of this country and in Europe where rubella-infected infants have been studied carefully.

The fact that the virus persists in the babies for so long is disconcerting. Of the 70 infants, 84 percent were positive; virus could be grown from the throat, from the urine, and from the rectum, but, peculiarly enough, not from the blood.

At autopsy, virus recovery was ubiquitous. Every organ cultured grew rubella virus. Thus these babies are born with a disseminated, diffused rubella virosis.

The children present an additional problem. An infant, born to a mother who had rubella in the first trimester, arrives in the nursery. Nurses, physicians, medical students, siblings, and incidental strangers are potentially exposed to rubella. To date, the graphed curve of viral shedding is a slow slope. The cases of some infants were followed for six months, nine months, and a year after birth, and were still shedding rubella virus. The public health problem is obvious. Since we have no protection against rubella embryopathy, the infant can be a potential source of infection to friends, relatives, and anyone with whom contact is made. There is no effective, practical solution to this problem at the moment. In every series of cases carefully studied, about 20 percent of the women during an epidemic year will contract rubella unknowingly. These women can deliver infants who will be shedding rubella virus. The mothers are not aware of it, nor are the physicians. The only possible way in which this enigma can be studied is by undertaking the expensive and difficult project of obtaining cultures on all newborn infants to detect those who have rubella. Where the mother is known to have had the disease or been exposed to it, her infant would be examined and cultures made. However, in at least 20 percent of the women there is no history. Thus one does not know whether he is dealing with a potential rubella infant or not.

We have discussed rubella, toxoplasmosis, salivary gland virus disease, and other viral bacterial or parasitic infections predisposing the mother to abortion, stillbirth, or neonatal disease but rarely associated with congenital malformations. There is a suspicion that other diseases probably have a teratogenic effect, but we do not have significant statistical evidence. For a time some

studies suggested that influenza (Asiatic influenza) was a teratogen. In at least two prospective studies this was refuted.

In the last few years it has been suggested that mumps contracted during early pregnancy might produce cardiac anomaly—fibroelastosis—in the fetus. This implication, too, is open to question.

Several years ago there was evidence that autoimmune disease in the human could produce teratogenic effects. The entity studied was thyroid disease—autoimmune thyroiditis. Studies indicated that cretinism (athyrotic cretinism) occurred in women who had a high titer of antithyroid antibody in their blood. Their infants also had a high titer. Unfortunately, further study did not substantiate the concept or the findings. In the future it is possible that more will be heard about autoimmune disease as a teratogen.

One of the problems we face in understanding teratogenicity in animal experimentation is that of prediction. The foremost problem is species variability. Cortisone produces cleft palate in mice and in rabbits but not in rats. Thalidomide is thought to be the most potent human teratogen; yet in other species it is difficult to produce malformations. Rabbits are susceptible, rats are not consistently so. We must appreciate and clearly understand that there is a specific and definite species variability in susceptibility and apparent immunity to teratogenic agents.

There also is a strain variability within the species, which compounds the problem. This problem of strain variability has been investigated in some detail by many scientists. The tabulation of cortisone-induced cleft palate comes from Kalter's work in Cincinnati. Strain A of mice will produce 100 percent offspring with cleft palate when the mice are injected with cortisone. The C-57 Bal strain will produce only 19 percent cleft-palate offspring. If a male C-57 is cross-mated with a female Strain A, 43 percent of the offspring will have cleft palate; if a female C-57 is cross-mated with a male Strain A, only 4 percent will have cleft palate. Hybridization within the species will produce a varying incidence of cleft palate. Some mice are totally impervious to the effects of cortisone. One can appreciate how this problem is multiplied when dealing with the human where the genotype is almost totally unknown and pretty regularly mixed.

A single agent can produce multiple malformations. There are many examples, the most obvious being rubella, that produce malformations of the eye, the heart, and the ear. Thalidomide produces a variety of malformations, as mentioned earlier. The same anomalies can be produced by a variety of teratogens. Cleft palate has been produced in the laboratory by cortisone, trypan blue,

vitamin A, and antifol. Exencephaly (a protrusion of the brain through a defect in the skull) has been produced by a variety of agents. To complicate the picture further, the genetic malformations are often indistinguishable from the environmental malformations. The phenomenon has been given the name "phenocopy."

The multiplicity of clinical manifestations in general makes it very difficult to arrive at a conclusion or opinion in the delivery room in terms of history. Organogenesis begins before recognition of pregnancy. It is completed in the human by 10 weeks. Obstetricians report that they rarely see a patient for the first time before she is at least two months pregnant. Thus prevention of exposure to noxious agents relative to the embryo requires not only education of the profession but also education of the laity. Women in their reproductive years are constantly exposed to a variety of nostrums and all sorts of over-the-counter preparations, which they may be ingesting before they know that they are pregnant.

Another difficult facet is our ignorance of low-incidence teratogens. It is hoped that this may be solved as we become more proficient and sophisticated in statistical acquisition and computerizing. If thalidomide had produced an incidence of only one or two percent malformations, there is a good probability that we still would not know about it. It is tremendously difficult to pinpoint a low-incidence teratogen unless you suspect and then pursue it. Otherwise, it very quickly becomes absorbed and lost to analysis within the total statistics of a studied area.

The inadequacy of malformation statistics becomes quite evident when one endeavors to compile data. There is no baseline standardization. Some statistics are taken from birth certificates. If any of you have had experience in delivery rooms where birth certificates are completed, you know how probable the errors can be. Other malformation statistics are obtained from hospital records and various public records, so that one set of statistics cannot be validly compared with another set. Finally, it has been shown conclusively that the incidence of malformations seen in the "newborn nursery" is probably one half of the total incidence. If the same infants are examined at six months and at one year, the number of malformations doubles. Many malformations are not discernible during examination in the "newborn nursery." Obviously, control studies are impractical, and one hopes that future teratogenic research in primates may give us clues and pertinent information, which present laboratory investigations are unable to do.