A HISTOLOGICAL AND HISTOCHEMICAL STUDY
OF THE DEVELOPMENT OF THE STERNUM
IN THALIDOMIDE-TREATED RATS

by
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INTRODUCTION

It is now well established that certain chemical compounds, administered to animals in early pregnancy, can adversely influence the development of the fetus, resulting in congenital malformations. Recently, it has been shown that thalidomide, a sedative drug, may induce skeletal defects in the offspring of treated females when administered in the early stages of pregnancy. McColl, Globus and Robinson (1963) reported that certain skeletal defects could be produced in the offspring of Sprague-Dawley rats following chronic oral administration of thalidomide. Notable among the defects produced were malformations of the sternum.

Previous investigations were confined, mainly, to describing the type and incidence of such malformations. For this reason, it was decided to investigate the development of the sternum, under the influence of thalidomide, so that a clearer understanding of the associated histological and histochemical changes could be achieved.

Most of the investigations into the mechanism of action of thalidomide have been directed toward studies of its structural similarity to normal metabolites, followed by testing of its anti-metabolic activity using in vitro systems. It was thought that a histochemical investigation of abnormal sterna might uncover a malfunctioning of one of the basic metabolic pathways, thus leading to the underlying cause(s) of the defects.
Although the anatomical and histological development of the mammalian sternum has been described (Paterson, 1900; Hanson, 1919; Gladstone and Wakeley, 1932; Bryson, 1945 and Chen, 1952), so far, no systematic account has been given of the associated histochemical changes and of how these are correlated with the histogenesis of the bone. In addition, most of the investigations have been concerned with the normal development of the mouse sternum or with a modified development due to the action of mutant genes. Although the development of the sternum in rats would not be expected to differ greatly from that in mice, seemingly minor differences between genera, even temporally, might be significant when comparing the development of normal and abnormal individuals. It was therefore decided to describe the normal development of the sternum in rats with emphasis on determining the principal events leading to the establishment of the normal segmented sternum.

Although the sternum has been shown to be largely self-differentiating, its segmentation is not controlled by intrinsic factors (Chen, 1953). That segmentation depends on the influence of the ribs is generally accepted and has led to the postulation of an inhibitory effect of the rib on chondroblastic hypertrophy in the sternal tissue. How it is produced remains obscure, and no conclusive evidence as to whether it is due to chemical or mechanical factors (or to both) has been provided. The effect of thalidomide on the morphology of the sternum is to alter the pattern of segmentation. It was, therefore, decided to pay particular attention to the segmentation of the sternum and especially to the histological and histochemical changes taking place in the sternocostal junctions.
It has long been known that one characteristic of the transformation of cartilage to the hypertrophic state, is the appearance of appreciable amounts of glycogen in the cytoplasm of the hypertrophic cartilage cell. Cartilage is a major repository of glycogen in the fetal body during the period of rapid skeletal growth (Gutman and Yu, 1950). The cytoplasmic deposits of glycogen, as can readily be demonstrated by histochemical methods, disappear just prior to or simultaneously with the appearance of bone salts in the intercellular cartilage matrix. In view of the proposed role of glycogen in the mechanism of endochondral calcification, it was decided to investigate the distribution of glycogen throughout the complete embryonic period of sternal development.

The association of alkaline phosphatase with the process of calcification in cartilage and bone has been confirmed by numerous workers since Robison (1923) first suggested it. It was shown that alkaline phosphatase was not present in cartilage which was not ossifying, but that at the stage of ossification which is represented by hypertrophy of the cells, the enzyme appeared. In view of its probable role in the mechanism of calcification and ossification, it was decided to investigate the distribution of alkaline phosphatase in the developing sternum of both normal and thalidomide-affected embryos.

The early investigations of Brachet (1947, 1950), Caspersson (1947) and others in recent years have resulted in the identification of cytoplasmic basophilia in many cell types. That these basophilic substances are due to the presence of ribonucleic acid (RNA) has received much support; that RNA is directly concerned with protein
synthesis has been universally accepted. It was, therefore, thought that an investigation of the ribonucleic acid content of the cells throughout the development of the sternum would be of particular interest in view of its probable role in the formation of the protein portion of cartilage and bone matrix.

Calcification of the cartilage matrix is correlated with the advance of the marrow in the process of replacement of cartilage by bones (McLean and Bloom, 1940). It is also correlated with the state of development of the cartilage cells, calcification occurring only in the zone characterized by cells described as hypertrophic. It was decided to investigate the time of appearance and extent of calcification in abnormal sterna and compare it with the normal pattern.

The object of this investigation is, therefore, threefold: (1) to study the histogenesis of the sternum in both normal and thalidomide-treated rats, paying particular attention to the problems of segmentation; (2) to study, histochemically, the distribution of glycogen, alkaline phosphatase, ribonucleic acid and calcium in the developing sternum of both normal and treated animals; and (3) to attempt an explanation of the underlying cause of the observed sternal malformations, based on the histochemical methods outlined above.
LITERATURE SURVEY

The Development of the Sternum

Early work on the development of the sternum was mainly concerned with the controversy over its origin. Paterson (1900) studied the early development of the sternum in rats and concluded that the sternum arises in association with the shoulder girdle - the coracoidal origin. Ruge (1880) and Mueller (1906) put forward a theory which derived the sternum from the ventral extremities of the ribs - the costal origin. Whitehead and Waddell (1911) favoured an "in situ" theory, suggesting that the sternum arises independently of either ribs or coracoid. The works of Hanson (1919), Gladstone and Wakeley (1932) and Chen (1952) all support the "in situ" theory.

The first experimental studies on the origin and development of the sternum were made by Fell (1939) in birds. Using the tissue culture technique, she showed that the avian sternum originates independently of both the ribs and the pectoral girdle, that the sternal rudiments are capable of considerable self-differentiation in vitro, and that the primary factor responsible for the median movement and fusion of the two bilaterally-situated rudiments is the migration of undifferentiated tissue in the dorso-lateral body wall.

Bryson (1945), made an embryological analysis of normal sternal
development in mice and compared it with the abnormal pattern as produced by the action of a mutant gene. He concluded that (1) increased width of the mutant sternum was not a primary effect of the gene, but was a secondary effect due to growth retardation of the ribs; (2) lack of segmentation and associated sternal width was a direct consequence of retarded rib growth and (3) segmentation of the sternum was the result of inhibition of cell hypertrophy, by the rib tips, at the sternocostal junctions.

Chen (1952) presented a systematic account of the histological changes associated with the development of the sternum in mice and intimated that the ribs may be concerned with the segmentation of the sternum. In a later investigation (1953), employing tissue culture techniques, Chen concluded that the effect of the rib stumps was to inhibit the hypertrophy of the cartilage cells adjacent to them, resulting in the formation of a transverse joint, and thus giving the sternum a segmental nature.

Malformations of the Sternum due to Thalidomide

Up to the present, no work had been done on the histogenesis of bone under the influence of thalidomide, although reports on the nature and incidence of skeletal malformations in animals and man have appeared in abundance.

Little attention had been given to sternal abnormalities induced by the drug until the work of McColl, Globus and Robinson (1963 and 1964). They observed the absence of ossification centers and a high incidence of "scrambled" sternebrae.

Obbink and Dalderup (1963) confirmed these observations and
reported an increased frequency of abnormality in the fifth ossification center of the sternum. They concluded that thalidomide had some influence on the ossification process in the rat.

Histochemical Studies on Developing Bone

It is beyond the scope of this investigation to survey all the literature on the histochemistry of developing bone. However, a number of excellent articles, reviews and books are available, which provide an accurate and exhaustive review of the subject (Pritchard, 1952; Bourne, 1956; Gibson, 1957; Neuman and Neuman, 1958; Rodahl, Nicholson and Brown, 1960; and McLean and Urist, 1961). The above authors review the roles of glycogen, alkaline phosphatase, ribonucleic acid and calcium in bone formation, which forms the basis of discussion for the present investigation.

Little work has been done on the histochemistry of the developing sternum and no work on the histochemistry of thalidomide-treated animals.
MATERIALS AND METHODS

Sprague-Dawley (CD) rats, obtained from Charles River Breeding Laboratories, were maintained on Purina Laboratory Chow until seven days prior to breeding.

The experimental group was subsequently maintained on powdered chow containing 2% thalidomide; the control group was maintained on powdered chow alone. Food was provided ad libitum for both groups. The treatment was continued throughout the period of gestation.

Seven days after commencement of the treatment, female rats, weighing approximately 200 grams, were mated with mature males. Vaginal smears were made daily and the day on which sperm was found in them was considered the first day of pregnancy.

The embryos were delivered by caesarian section on successive days beginning on day 12 of gestation and continuing up to the time of birth (day 23).

Preparation of Material for Whole Mounting

Full-term embryos were fixed in 10% formalin for a period of from 1-2 weeks. The skeletons were subsequently stained using the method of Dawson (1926) modified as follows: 10% formalin rather than 95% alcohol was employed as the fixative. The specimens were cleared in a 10% aqueous solution of KOH for 5-7 days. Use of a 1% solution of potash as described by Dawson, takes 12-14 days and was found to render the tissue more liable to maceration.
Preparation of Material for Serial Sectioning

Embryos 12-16 days old were decapitated coincident to the removal of caudal somites. The remaining trunk portion was then transferred to the various fixatives employed in this investigation.

In embryos 17-22 days old, the sternum was excised as a rectangular strip of tissue with the rib tips intact so that their articulation with the sternum could be visualized. Immediate transfer to the fixatives was effected without delay.

The fixatives were selected according to the staining methods subsequently to be employed and included aqueous Bouin's Fluid, 80% alcohol, Carnoy's Fluid, Rossman's Fluid and 10% neutral formalin. The tissues were not decalcified as the embryos to be sectioned did not exceed 23 days of age. (Mineralization at this stage is not extensive enough to prohibit sectioning).

All embedding was done in paraffin and sections were cut in frontal plane at 5-8 \( \mu \) in thickness.

Histology

The general histology was studied in preparations stained with Harris hematoxylin and eosin B. Staining of collagen fibers was achieved using (1) the Aniline Blue Collagen Stain of Mallory, 1938 (in Davenport, 1960) and (2) iron hematoxylin of Heidenhain followed by van Gieson's stain, (in Carleton and Drury, 1957). H. R. Scott's modification (1952) of the aldehyde fuchsin technique of Gomori, was employed for the purpose of differentiating between the ground substance of cartilage and bone matrix. The technique involves the use of three stains - aldehyde fuchsin, phloxin and fast green. The
ground substance of cartilage exhibits the purple coloration of the aldehyde fuchs in while the bone matrix is stained green.

Histochemistry

1. Alkaline phosphatase activity was investigated by Gomori's method (1952) after fixation in 50% alcohol. In order to reduce diffusion of the enzyme to a minimum, the time of incubation was kept as short as possible. Thus incubation times of 20 minutes to 1/2 hour were employed as a routine. In order to remove preformed calcium salts from the bone matrix, the sections were treated for 15 minutes with a citrate buffer solution prior to incubation. Control slides were obtained by omitting the glycerophosphate from the incubating medium but were otherwise treated as described above.

2. Glycogen was demonstrated after fixation in Rossman's Fluid and stained by the Periodic acid - Schiff technique (McManus, 1948). Schiff's reagent was prepared according to de Tomasi (1936). Optimal staining was achieved after 15 minutes in Schiff reagent. Control slides were treated with malt diastase at 37° C, for two hours. Glycogen was differentiated from other materials, particularly the glycoproteins of cartilage matrix, by use of the control sections exposed to the action of malt diastase, which digests out glycogen leaving a negative image of the substance.

3. Cytoplasmic basophilia, due to the presence of ribonucleic acid (RNA), was demonstrated by the pyronin Y-methyl green method of Kurnick, 1955 (in Pearse, 1960). Fixation was effected using Carnoy's Fluid and the duration of staining was adjusted at 30 minutes. This combination stains the basophilic material of the cytoplasm and the
nucleolus, red, and that of the nucleus, bluish-green.

4. Sites of calcium deposition were demonstrated with von Kossa's method, 1901 (in Pearse, 1960). The tissues to be studied were fixed for 24 hours in 10% formalin, neutralized by saturation with MgCO₃. The tissue is first impregnated with AgNO₃ for one hour, in the dark, with subsequent reduction of the silver salt by ultraviolet light for 20 minutes.
OBSERVATIONS

Histology

Development of the Sternum - Normal

The development of the sternum in the rat is not dissimilar from that of the mouse (described by Bryson, 1945; Chen, 1952), however, the appearance of the various stages of development are not contemporaneous in the two genera. It therefore seems appropriate to describe briefly the development of the sternum in the rat (1) for the purpose of comparison with the thalidomide-treated group and (2) to serve as a framework for discussion of the histochemical changes associated with the development of both normal and abnormal sterna.

The emphasis will be placed on the later stages (17 days onwards) since most of the significant findings occurred after the appearance of the definitive cartilage. The ribs are first recognizable in a 13-day embryo. They appear as loose, rod-like condensations of mesoderm, growing out in the myosepta of the thoracic vertebrae. They extend only a short distance laterally where they end in a blunt point. No sign of the sternum can be seen at this time. By the fourteenth day the ribs are clearly distinguishable from the surrounding tissue and have moved further laterally and slightly ventrally.

The paired sternal anlagen make their first appearance on or
slightly before the fifteenth day, arising immediately below the ventral ends of the ribs. They are first distinguishable from their articulation with the clavicles, backwards to about the level of the first sternal rib, and appear as paired mesodermal condensations, the boundaries of which are difficult to define.

The paired elements of the future sternum are at first widely separated by the pericardial cavity and are completely independent of the ribs with which they will later come into association.

By about the sixteenth day of embryonic life, the paired sternal primordia have united anteriorly, resulting in the formation of a "V", with the apex pointed anteriorly. At this stage, a close association exists between the sternal bands, which are composed of precartilage (figure 3), the clavicles, and the rib tips, which have chondrified. (In the normal rat there are seven ribs articulating with the sternal bands). From the level of the first sternal rib caudalwards, the sternal bands diverge laterally.

Migration of the sternal rudiments ventro-medially brings them in close approximation to one another in the midline and by the seventeenth day, only a narrow zone of loosely arranged tissue separates them (figure 4).

By the eighteenth day, fusion of the sternal bands is complete, forming a continuous bar of cartilage with all the principal anatomical outlines of the adult structure (figure 6). The rib tips are bilaterally opposed with layers of flattened chondroblasts concentrically arranged around the penetrating rib tip. In the intervening tissue of the sternocostal junction, the cells remain small and there is very little basophilic matrix. The intercostal
regions at this stage resemble closely the structure of the rib tip, but the former soon undergoes hypertrophy, whereas the rib tip retains its characteristics and ceases to advance.

At twenty days, chondroblastic hypertrophy is well advanced in regions corresponding in location to the areas of future bony elements of the sternum (figure 8). Collagen fibers, demonstrated by aniline blue staining, are interspersed between the hypertrophic cells in the intercellular matrix, in the matrix of the rib tips, in the zone of proliferation of the intercostal segments and in the layers of flattened chondroblasts surrounding each rib tip, but very little collagen fiber formation is evidenced in the intervening tissue of the sternocostal joints.

Typically, there are five articulations located at the sternocostal junctions, separating six centers of ossification, i.e. the manubrium, four sternebrae and the xiphisternum, each corresponding to one of the areas of hypertrophic cartilage. Since segmental hypertrophy preparatory to ossification occurs at different times in different segments of the sternum, it was thus found more practical to describe the development of one center (the third) and to restrict all further observations to the one element.

At the twenty-first day, ossification has begun within the third segment, the details of bone formation following the classical scheme beginning with periosteal ossification, and ultimately forming both periosteal and endochondral bone. The hypertrophic cartilage soon becomes calcified (figure 47) and the eroded cartilage is invaded by vascular mesenchyme which penetrates the periosteal bone (figure 9). At 22 days, several zones can be identified and defined
according to the state of the cells: (1) a central core of eroded cartilage; (2) above and below this a zone of hypertrophic cartilage cells with vacuolated cytoplasm; (3) next to which is a zone of compressed cells undergoing proliferation and (4) a collar of periosteal bone (see figure 10).

Normal bone growth continues, interrupted only in the region where ribs and sternum come into contact at the site of the future sternocostal joints. In the normal sternum the last element to undergo ossification is the fifth.

By the time of birth (23 days) the sternum exhibits six, well-ossified segments. At the sternocostal junctions, two transverse bands of cells are seen to unite with the regions of concentrically oriented cells next to each rib tip. The chondroblasts, which are arranged in layers at right angles to the long axis of the sternum, nearly eliminate the central space which was originally filled with loosely scattered cells. This observation has been interpreted by Bryson, (1945) and Chen (1952) to represent an early stage in the formation of a transverse joint at the level of each pair of ribs.

By the twenty-third day, the medullary cavity of the sternebra is composed principally of cancellous bone. Numerous osteoblasts are seen lining the borders of newly-deposited endochondral bone.

Development of the Sternum - Thalidomide-treated Group

In the thalidomide-treated group, mesenchymal condensations, corresponding to the future ribs, can be demonstrated in a 13-day fetus, but the ribs are less densely arranged than those of the control group. Similarly, the sternal primordia are in the form of
discernible mesenchymal condensations by the fifteenth day, but the extent of aggregation of the cells suggests a retardation in development.

At the sixteenth day of gestation, the paired primordia are still widely separated by the pericardial cavity, union of the sternal bands not having occurred. The delay in development at this stage may be estimated to be 12-18 hours. The difficulty in ascertaining an estimate of the delay may be attributed to the problem of determining the precise time of conception.

By the seventeenth day, the sternal bands have united at the anterior end but a wide zone of loosely arranged tissue still separates them. From the level of the fourth sternocostal junction caudalwards, the bands diverge laterally (figure 5) unlike the control sterna which had come into close association at this time (figure 4). (When compared to controls of the same age, an estimated delay in development of twelve hours was apparent.) Bilateral asymmetry of sternocostal articulations, a factor which has been shown to affect the future pattern of development of the sternebrae (Bryson, 1945), can be seen in figure 5.

Fusion of the bands has taken place, in 18-day embryos, down to the level of the fourth sternocostal junction, but posterior to this, a narrow gap still separates the members of the pair of sternal bands (figure 7). Bilateral asymmetry of sternocostal articulations may again be observed in addition to which there is some degree of torsion in the sternum, presumably resulting from uneven pressures exerted on the sternum by misplaced rib tips.

Bilateral asymmetry of sternocostal articulations was seen to
be a fairly constant feature in the drug-treated group and was observed at all stages of development from day 17 to day 23.

Chondroblastic hypertrophy was evident in 20-day embryos, but in about 75 per cent of cases, only five centers were observed as compared to six in control animals. Occasionally, two centres of hypertrophy appeared in one segment, indicating the dual origin of the ossification center.

Histologically the cartilage cells were more compressed at the sternocostal junction of normal as compared with the drug-treated group.

Collagen fiber formation did not appear to be affected in the drug-treated group. Slides stained with Mallory's aniline blue collagen stain were not noticeably different from controls, at the same histological stage of development.

In twenty-one day embryos, periosteal bone formation had begun and by the twenty-second day, periosteal membrane bone was well advanced. In 23-day embryos the medullary cavity of the sternebra was composed principally of unresorbed cartilage spicules instead of cancellous bone as in the normal sternebrae. Osteoblasts were numerous as in the normal, but endochondral ossification appeared to be decreased, whereas periosteal ossification seemed to be normal.

Macroscopic Observations

Changes in Costal Cartilage

Number of Ribs Articulating with the Sternum. In all control
embryos studied, thoracic ribs numbers 1-7 are "true" vertebro-sternal ribs, i.e. they make intimate contact with the sternum (see figure 11).

In the treated groups, considerable variation was found to exist. In some, the last rib to articulate was number 6 (figure 12). In others there were seven costosternal articulations, but ribs numbers 5-7 were crowded together (figures 20 and 21), all three ribs articulating between sternebrae numbers 4 and 6 (number 5 is missing).

**Pattern of Articulation between Ribs and Sternum.** Irregular shifting of costosternal junctions was frequently observed in the treated group (figures 13, 14, and 15). Abnormal spacing between consecutive ribs was a constant feature (figure 15). The rib tips in these individuals were not bilaterally opposed, as unilateral or bilateral shifts in the costosternal margins had taken place. This gives rise to the abnormal and variable patterns of ossification of the sternum.

Sternal Abnormalities

**Absence of Sternebrae.** Absence of sternebra number 5 was frequently observed in association with a reduction in "true" ribs to six in number (figure 12), or a crowding of ribs numbers 5-7. The inhibiting effect that crowding produces upon ossification is seen in figures 20 and 21.

**Asymmetry.** Asymmetry of sternal elements occurs with high incidence and great variation in design. The basic change appears to be the fusion of one lateral half of a sternebra to the opposite half of the sternebra anterior or posterior to it (figure 15). In less extreme cases one lateral half of a sternebra has shifted slightly
anteriorly or posteriorly and has been shown to be related to the pattern of costosternal articulation (Chen, 1952; Bryson, 1945).

**Dual Ossification Centers and Bifurcation.** Centers of ossification frequently arise as two distinctly separate halves of one sternebra. At 23 days gestation, the two halves have not fused together to form a unit, but exhibit a characteristic median division (figure 16).

Bifurcated sterna are less frequently encountered, but not uncommon. In these individuals the sternum is cleft down to the level of the second or third sternebra and occasionally throughout its entire length, (figures 17-21). Usually associated with this condition is a missing fifth sternebra in each half of the sternum and crowding of the fifth to seventh costal cartilages. The overall length of the sternum is reduced and each half is as wide as a normal whole sternum. Ossification of the sternebrae proceeds in spite of the failure of the two lateral bars to reach the midline and fuse.

**The Influence of the Ribs on Segmentation of the Sternum.**

Normally the onset of ossification within the sternum of mammals occurs midway between the sternocostal junction of one pair of ribs and an anterior or posterior pair (Bryson, 1945). The sphere of influence of the rib tip appears to extend from the point of contact between rib and sternum to the midpoint of the latter.

As described above, unilateral or bilateral shifting of sternocostal articulations was seen to occur in the drug-treated group. Inhibited areas of ossification are thus distributed in
secondary conformity to the new pattern of sternocostal articulation (figures 13-15).

Histochemical Observations

Glycogen - Control Group. The seventeenth day of gestation marked the appearance of glycogen in the chondrocytes of the developing sternum. The chondroblasts exhibited a negative staining reaction for glycogen while the prechondroblasts were intensely stained. At this stage a negligible amount of glycogen was present in the rib stumps still composed of immature cartilage. A high content of glycogen was seen in the prechondroblasts but no glycogen storage could be demonstrated in the chondroblasts of earlier stages.

As the cells enlarged and matured, intense cytoplasmic storage of glycogen was observed in the intercostal segments of the sternum. The stages of intense glycogen storage corresponded to days 18-20 of development (figures 22-23). The glycogen content of the cells increased markedly in the zones of hypertrophy and proliferation during the nineteenth and twentieth days of gestation, whereas storage of glycogen in the small-celled cartilage of the sternocostal joints was not noticeably augmented (figure 22). (Storage of glycogen is associated with the approaching hypertrophy of the chondrocytes and reaches its maximum in the hypertrophic cells.)

At twenty days gestation, glycogen completely filled the cytoplasm of the hypertrophic cells.

Glycogen storage remained intense in the early stages of cell hypertrophy prior to endochondral ossification, but as erosion of the cartilage model took place and pre-osseous matrix began to calcify
(parallel series stained for calcium), glycogen storage dropped significantly.

The central eroded area of hypertrophic cells was completely depleted of glycogen by the twenty-first day of gestation with the exception of intact cells at the borders of the zone of erosion (figure 26). The periosteal osteoblasts displayed intense glycogen storage at this stage. The glycogen content of osteocytes embedded in the matrix of the periosteal collar was characteristically negligible by the twenty-second day (figure 32).

At 22-23 days gestation, barely a trace of glycogen could be found in the hypertrophic cartilage (figures 28, 30 and 33), the extent of which was limited to a few rows of cells adjacent to the zone of proliferation. At the 23 day stage, primary spicules of endochondral bone had been laid down in the eroded area (figures 33 and 35). The medullary osteoblasts, distributed along the borders of primary spicules of endochondral bone, displayed a more intense staining than those osteoblasts associated with the periosteal collar. Osteocytes, embedded in the matrix of the newly formed spicules of endochondral bone, displayed a characteristically low content of glycogen.

Glycogen - Treated Group. Evidence of retardation in development was afforded by the delay in the appearance of glycogen storage in the sternum of the treated group. No glycogen storage was seen before the eighteenth day of gestation (about one day late). The pattern of storage, namely heavy deposition in the prechondroblasts, decreasing to none in the chondroblasts, with a reappearance of
glycogen in the chondrocytes, was not dissimilar from that observed in the controls.

By the nineteenth day, heavy stores of glycogen were present in the intercostal segments of the sternum (figures 24 and 25) and through to day 20 no remarkable differences, from control, were observed.

Striking differences were noticed in embryos, 21-23 days old. At 21-22 days large stores of glycogen granules were seen to occupy the lacunae of disintegrating hypertrophic cartilage cells in the zone of erosion (figures 27, 29 and 31). Cytoplasmic storage was maintained in the still intact cells so that at the time of disintegration, a large number of granules of glycogen can be visualized. At 23 days gestation, glycogen storage in the hypertrophic cells was still intense (figures 34 and 36). Spicules of bone had formed but they were irregularly distributed and much smaller than those formed in control animals. Osteoblastic activity was not different from control.

Alkaline Phosphate - Control. Alkaline phosphatase activity is not found in association with the formation of cartilage unless hypertrophic changes supervene (Pritchard, 1952). Accordingly, no appreciable activity was observed in the costal cartilages at any stage of development. Alkaline phosphatase activity was first detected in small amounts in the sternum in the intercostal segments of 20-day embryos. The cartilage constituting the sternum at stages earlier than this was entirely devoid of the enzyme.

At 21 days gestation, enzymatic activity was intense in the
hypertrophic cartilage cells, periosteal osteoblasts and invading vascular mesenchyme (figures 37 and 39). The intercellular matrix and periosteal osteocytes also gave positive reactions but of lesser intensity. Phosphatase activity at this stage was confined to the first two or three rows of cells in the zone of proliferation and was absent from the small-celled cartilage of the sternocostal junction (figure 37).

At 22 days, the hypertrophic cells and periosteal osteoblasts were still strongly positive but activity was confined mainly to the cytoplasm. Fine granules were visible in the intercellular matrix, indicating the presence of enzyme.

The zone of hypertrophy which had been reduced to about four cells in thickness was still at a peak of activity at 23 days gestation (figures 40 and 43), and large granules of precipitate were seen in the intercellular matrix. Both periosteal (figure 45) and medullary osteoblasts (figure 43) exhibited intense activity as both periosteal and endochondral bone was being formed. The osteocytes, at this stage, were almost devoid of the enzyme.

Alkaline Phosphatase - Thalidomide-treated Group. In the treated group, an atypical distribution of alkaline phosphatase was observed in the sternebrae undergoing ossification.

At 21 days gestation, no activity could be detected in the hypertrophic cartilage cells or intercellular matrix, but some periosteal osteoblastic activity was observed (figure 38).

At 23 days gestation, only scattered hypertrophic cells showed phosphatase activity and those that did exhibited a weak reaction
(figures 41, 42 and 44). Large quantities of phosphatase could be seen in the periosteal osteoblasts and vascular mesenchyme and accordingly, periosteal bone formation appeared to proceed along a normal course (figure 46). However, bone spicules of endochondral origin were retarded in their development.

**Calcium - Control Group.** The presence of calcium deposits in the developing sternum, as shown by the silver nitrate technique, was demonstrated on the twenty-first day of gestation. The first demonstratable bone salt appeared as fine brownish granules or as larger brownish-black aggregates scattered in the cartilage matrix. Judging from the extent of salt deposition calcification of the cartilage matrix began on the twenty-first day, accompanying the erosion of the cartilage and penetration of vascular mesenchyme. The extent of calcification can be seen in figure 47.

By the twenty-second day of gestation, and subsequent to the erosion of the calcified cartilage, trabeculae of bone had been laid down in the primitive marrow cavity. These bone spicules were heavily infiltrated with bone salt. The extent of calcification at this stage can be seen in figure 48. Periosteal bone was well calcified at this stage.

By the twenty-third day of gestation, heavily calcified trabeculae of bone were seen to project into the intercostal segment with an orientation in the plane of the long axis of the sternum (see figure 49).

**Calcium - Treated Group.** The presence of calcium salts was shown at 21 days gestation but the distribution and degree of
calcification was not as extensive as in control groups.

By the twenty-third day, heavy deposits of calcium were observed, but there were numerous centers of crystallization, most of which were extremely small and disoriented (figure 50), in marked contrast to the controls (figure 49). These small deposits of calcium, principally confined to the center of the sternebra, probably represent unresorbed spicules of cartilage impregnated with calcium salts. As was the case in the control group, periosteal bone was well calcified at this stage.

**Ribonucleic Acid - Control Group.** Cytoplasmic and nucleolar basophilia, demonstrated with pyronin stain, is associated with its ribonucleic acid content (Pritchard, 1952). As the cartilage cells of the sternum matured, an increase in cytoplasmic basophilia was observed. At the definitive chondroblast stage, a decrease in the ribonucleic acid content of the cytoplasm was observed, and with the onset of hypertrophic changes in the intercostal regions of the sternum, cytoplasmic basophilia was lost (figures 53, 55 and 57). At all stages of development, after the establishment of the final form of the sternum, the small-celled cartilage of the sternocostal joint exhibited an intense staining reaction to pyronin (figure 51). The same was true in the rib tip, particularly in the concentrically-arranged, flattened cells around the tip of the penetrating rib.

At the twenty-first day of gestation, intense staining of the periosteal osteoblasts was observed, while cytoplasmic basophilia of the hypertrophic cells was all but abolished (figure 55).

By twenty-three days gestation, osteoblastic activity was
maximal, as numerous medullary osteoblasts, with intensely-stained cytoplasm and nucleolus, were seen to line the borders of the now enlarged spicules of bone (figure 59).

**Ribonucleic Acid - Treated Group.** Throughout the period of gestation, no deviation from control was observed. The staining of cytoplasmic and nucleolar basophilia (figures 52, 56 and 60) paralleled that seen in control animals (figures 51, 55 and 59).

**Explanation of Histogram**

Figure 1 is a histogram, showing the relative amounts of glycogen and alkaline phosphatase present in the hypertrophic cartilage cells and the relative amounts of calcium present in the intercellular matrix of the developing sternum, on each day of embryonic development from day 17 to day 23. A comparison is given of the relative amounts of each substance in both thalidomide-treated and control groups. The intensity of the stain is taken as an indication of the presence of larger or smaller amounts of the substance studied.
FIGURE 1

Legend:

- Glycogen
- Alkaline Phosphatase
- Calcium

THALIDOMIDE

CONTROL

DAYS OF GESTATION
Sternocostal Junctions

As previously noted, the cells adjacent to the rib tip are compressed and concentrically arranged around the penetrating rib tip. In the center of the junction, the cells are very loosely arranged, with very little basophilic matrix in the intercellular spaces. At 17 and 18 days gestation, all of the cartilage cells comprising the sternum possess cytoplasmic stores of glycogen, but shortly afterwards (19 and 20 days gestation) the glycogen stores in the junction are depleted, while storage in the intercostal region is enhanced. Alkaline phosphatase activity is not observed in cells of the joint at any time. On the other hand, the ribose nucleic acid content of the cells comprising the joint is considerable.

From 21 to 23 days gestation, a number of changes can be observed. The concentric layer of flattened cells surrounding the rib tip exhibit a large number of mitoses and elongated cells, oriented transversely to the long axis of the sternum, can be seen streaming toward the center of the joint. The cells appear to be originating from the rib tip itself, replacing the pre-existing, loosely-arranged connective tissue of the joint. Beginning at about 21 days, cytoplasmic storage of glycogen is again observed as well as an increase in basophilic matrix. The cells contain large amounts of ribose nucleic acid but no alkaline phosphatase activity is observed. By the twenty-third day of gestation, the transverse bands of cells uniting the opposing rib tips nearly eliminate the central space, previously characterized by signs of inactivity or degeneration. A decrease in glycogen storage and an increase in cytoplasmic basophilia is observed.
DISCUSSION

The Influence of the Ribs on Fusion and Segmentation of the Sternal Bands.

The histological study of the normal and abnormal development of the rat sternum furnishes further evidence in support of Bryson's (1945) and Chen's (1952) observations suggesting that the ribs are concerned with the segmentation of the sternum. The present investigation has demonstrated that whatever the pattern of sterno-costal articulation, the sternebrae hypertrophy and subsequently ossify in secondary conformity to the original developmental error, that is maldevelopment of the ribs.

A dependence on normal rib growth is of fundamental importance in the determination of sternal pattern. Reduced and irregular intercostal spacing leads to asymmetry of sterno-costal articulations. Crowding of ribs ultimately leads to reduction in number of sternebrae (usually numbers 5-7 are packed together), leaving no space for intercostal segments to appear. Equally important is the elongation of the sternal rudiments and their ventral migration to finally fuse in the mid-ventral line. Failure of the sternal primordia to elongate results in only six sterno-costal articulations and five sternebrae or alternatively, if rib crowding accompanies failure of the sternal bands to elongate, then seven sterno-costal articulations will be formed, but only five sternebrae.
Uncertainty arises in the case of bifurcation of the sternum, since conflicting viewpoints as to the cause have been suggested. According to Bryson, sternal union at the midline is dependent upon rib growth while it is Chen's contention that the primary factor causing the union of the sternal bands is the streaming of undifferentiated cells from the dorso-lateral body wall in a ventral direction. Chen (1953) observed the effect of removal of the ribs on the development of the sternum in vitro, and found that the sternal bands moved medially toward each other and fused. As compared with sternal bands with ribs attached, the rate of movement seemed to be slightly faster in the explants without ribs. These facts suggest that the ribs retard the movement of the sternal bands, and therefore retarded rib growth may restrict the ventral movement of the sternal bands and prevent their fusion.

An examination of the sternum in figures 4, 5, reveals a lateral tension at the level of the first two ribs. Lateral drawing out combined with abnormal width of the sternal bands in this area indicates a struggle between opposing forces, namely movement of the sternal bands medially and restriction of movement by the growth-retarded ribs.

Additional support for the restricting effect of the ribs on sternal fusion is provided by examination of the cells adjacent to the penetrating rib tip. As stated in the observations, the cartilage cells are more compressed at the sternocostal junction of normal as compared with the drug-treated group. That these cells, in the drug-treated group, are not as compressed as normal, would appear to indicate retarded rib growth. Chen's observations suggest
that the migration and fusion of the sternal bands does not depend on pressure exerted by the ribs. Although the layers of compressed cells indicate that pressure is being exerted, the compressive effect is not observed until sometime after fusion has taken place. (It will be shown later that the compressive effect is more likely associated with segmentation rather than fusion of the sternal bands.)

A reasonable explanation of the effect of the ribs on fusion of the sternal bands may then be proposed; movement of the sternal bands does not seem to depend on pressure exerted by the ribs, but a restriction can be placed on sternal migration by growth retarded ribs.

Bifurcation of the sternum was invariably associated with (1) failure of the sternal primordia to elongate to normal proportions and (2) a decreased distance between ribs resulting in crowding. These observations suggest that both rib and sternal abnormalities originate through a general mesodermal growth deficiency which produce a modification of sternal pattern, indirectly, by way of the ribs.

**Delay in Development**

Histologically, the delay in development of the thalidomide-affected sterna, never exceeded twenty-four hours and was usually in the range of 12-18 hours. This would indicate that the delaying factor(s) having operated (probably during the period of major organogenesis) ceased to have any effect on subsequent events. These facts can be interpreted to mean that the onset of development of the sternum and not the developmental rate is retarded.
The effect is, however, more than an initial simple delay. Thalidomide influences not only the early chondrification center, but also the much later stages of hypertrophic cartilage. It influences glycogen, alkaline phosphatase and time of calcification at the later stages. There is, therefore, an initial delay but later influences can be described more specifically.

**Sternocostal Junctions**

As previously noted, Bryson (1945) and Chen (1953) suggested that the ribs are concerned with the segmentation of the sternum, and that the ribs may inhibit the hypertrophy and ossification of the cells immediately adjacent to them (that is, in the intercostal joint). The results of this investigation amply justify the thesis that the ribs are concerned with the segmentation of the sternum, but the reason(s) for the failure of the tissue in the joint to ossify remains uncertain. The "inhibitory effect" has been shown by Chen (1953) to be quite localized and has been produced in tissue culture by the addition of extra rib stumps.

Chen (1953) attempted to isolate a chemical factor that may be responsible for the inhibitory action of the rib stumps, but his attempts proved futile. He then set about trying to assess the possible influence of mechanical pressure exerted on the sternum by the penetrating rib tips. In these in vitro experiments, he substituted various other materials and tissues in place of the ribs, and observed no inhibitory effect on the cartilage cells of the sternum. Chen concluded that the effect may be chemical, mechanical, or both.
Although a number of cartilaginous tissues are known to be non-ossifying, the sternum has been shown to be an ossifying tissue. When Chen (1953) cultivated the sternum of mice in vitro, in the absence of rib tissue, the entire sternum underwent hypertrophy and subsequently ossified. As mentioned before, the failure of ossification observed at the sternocostal junction appears to be due to the influence of the ribs. Previously it was thought to be a question of which factor was responsible for the inhibition of hypertrophy at the joint. Perhaps inhibition is a misnomer and it is simply a matter of the replacement of degenerating tissue by non-ossifying rib cartilage, as the results of this investigation seem to indicate.

In this investigation, a histological and histochemical study of the cells comprising the sternocostal junction was undertaken in the hope that some additional light might be cast on the mechanism of inhibition.

The observations suggest that degeneration is taking place in the joint, at 19-20 days gestation, possibly due to pressure exerted on the cells by the penetrating rib tip. Chen (1953) was not successful in his attempts to inhibit the ossification of the sternum when glass capillary tubes or clavicles were substituted for ribs. It appears necessary to have a proliferating tissue growing into the sternal cartilage in order to create sufficient pressure to cause degeneration. Neither the glass capillary tube, the clavicles (which incidentally had already ossified) nor the dead rib tips satisfy the requirements of a tissue with terminal proliferation, creating pressure on the adjacent tissue.

Subsequent to the replacement of the degenerating tissue of the
joint by hyaline cartilage, derived from the rib tips (21 days onward), the newly-formed cartilage displays all the histochemical characteristics of the rib tissue, namely an increase in basophilic matrix in the intercellular spaces, an accompanying decrease in glycogen storage, an increase in the ribonucleic acid content of the cells and an absence of alkaline phosphatase activity. The decreased glycogen storage and increased cytoplasmic basophilia are probably associated with the elaboration of the cartilage matrix.

Previous investigators placed the emphasis in their investigations on the identification of a factor which could explain the failure of potentially ossifying tissue (the sternum) to ossify. The results of this investigation suggest that the potentially ossifying tissue is replaced by the proliferation of non-ossifiable cartilage. This hypothesis could be tested using tissue culture techniques and radioactive isotopes such as tritiated thymidine which would be incorporated into the DNA of the nucleus. By grafting labelled rib stumps to unlabelled sternal bands and following the distribution of labelled cells by autoradiography one can determine whether the cells of the sternocostal joint are replaced by proliferation of non-ossifiable rib cartilage. Such a result would probably prompt a restatement of the problem to read, "What is the basic biological difference between ossifiable and non-ossifiable cartilage?"

Mineralization

Glycogen

The transition of cartilage to the hypertrophic state has been
known for a long time to be parallel with the accumulation in the cytoplasm of the chondrocytes of large amounts of glycogen, which disappears either just before or simultaneously with the appearance of mineral salt in the adjacent cartilage matrix (Dixon and Perkins, 1956). "The formation and rapid utilization of such stores of intracellular glycogen would imply the presence within the calcifying cartilage cells of the entire complement of enzymes now known to participate in the synthesis and phosphorolytic degradation of glycogen" (Gutman and Yu, 1950).

Harris (1932) postulated a connection between glycogen and calcification suggesting that hexosephosphoric esters formed in the course of glycogen degradation might serve as substrates for cartilage alkaline phosphatase, in accordance with the Robison theory of calcification. The discovery by Gutman and Gutman (1941) of a phosphorylase in calcifying cartilage which could convert glycogen into hexosephosphate, has tended to support Harris' hypothesis. This enzyme initiates phosphorylative glycogenolysis by catalyzing the breakdown of glycogen and the formation of glucose 1-phosphate. Upon its discovery in calcifying cartilage, it was believed to lead to the synthesis of potential substrates for phosphatase in zones of calcification before blood sources become available, and to supplement these blood sources thereafter. (See McLean and Urist, 1961.)

The main pathway of phosphorylative glycogenolysis is illustrated in figure 2, beginning with the phosphorylation of glycogen to form glucose 1-phosphate, and continuing down to the formation of lactate. The steps indicated with solid arrows have been verified
Figure 2. Schematic representation of the role of phosphorylative glycolysis in calcification and mucopolysaccharide synthesis. Alternate pathway, starting at glucose, is indicated.
for calcifying cartilage, (McLean and Urist, 1961).

Recapitulating the findings of the present investigation, large stores of glycogen were seen to accumulate in the cytoplasm of maturing cartilage cells, especially in the region of hypertrophy. In the drug-treated group, large stores of glycogen granules were seen to occupy the lacunae of disintegrating, hypertrophic cells at a time when calcium salts were being deposited in the intercellular matrix. That is, the cells disintegrate before having metabolized the glycogen stores, leaving large amounts in the lacunae. This observation is in marked contrast to the normal pattern where rapid depletion of glycogen reserves was recorded.

In view of the proposed role of glycogenolysis in the calcification of cartilage matrix, that is, providing hexosephosphates necessary for mineral salt formation, it seems likely that an interference with glycogen metabolism would result in decreased deposition of mineral salt in the cartilage matrix. Calcification did occur in the drug-treated group, but to a lesser degree than in the control group. Only small centers of crystallization were observed which were of a less dense nature than those observed in the normal group. The formation of the ground substance may be affected by drug treatment with the result that there is retarded formation of the matrix. However, that matrix which is formed appears to become calcified, normally.

A rapid disappearance of glycogen from the hypertrophic cells, seen to occur in the controls but unparalleled in the drug-treated group, is suggestive of an inhibition of glycogen degradation and utilization in the abnormal embryos. Another interpretation could
be given to the persistence of glycogen storage in abnormal embryos, namely, that the rate of synthesis of glycogen may be increased and consequently larger stores are available for utilization. The latter seems less probable since the earlier stages of glycogen storage (18, 19 and 20 days gestation) run approximately parallel courses in both groups, and increased storage in abnormal embryos is not indicated.

Inhibitors of muscle glycogenolysis have been shown to block in vitro calcification of cartilage. Phlorizin, for example, inhibits cartilage phosphorylase (Gutman and Gutman, 1941) and in its presence, calcification in vitro fails when phosphorus is supplied as inorganic phosphate and only succeeds when supplied as glucose 1-phosphate, glucose 6-phosphate, fructose 1, 6-diphosphate or 3-phosphoglycerate - all phosphoric esters which appear in the cycle after the site of phlorizin block.

On the basis of the histochemical observations made in the present investigation, a block at A (the site of phosphorylase activity), due to thalidomide or one of its metabolites, is suggested (see figure 2). As in the case of phlorizin, the block may be partial but nevertheless, an obstruction. Although a number of histochemical changes were observed in the treated group, which could ultimately reduce the concentration of phosphates necessary for calcification, the cartilage matrix was still seen to calcify. A second mechanism for providing an increased concentration of phosphate may account, in part, for the above observation. Levine and Follis (1949) demonstrated the presence of a lecithinase in cartilage which will break down lecithin into a diglyceride and
phosphocholine; the latter is assumed to be broken down by alkaline phosphatase, providing an increased concentration of phosphate in the zone of provisional calcification of cartilage.

Mention has been made of the apparently normal development of periosteal bone, accounting for most of the bone material laid down in the abnormal sternum, while endochondral bone formation appears to be significantly retarded. This observation may be explained partly on the basis of the regional availability of blood supplying the tissues. Periosteal bone forming elements are richly supplied with blood, and thus with phosphate esters contained in the red blood cells (Dixon and Perkins in Bourne, 1956). In the formation of endochondral bone, the salts are first deposited in the so-called "provisional calcification zone" of the cartilage, beyond the disintegrating cartilage cells. This layer is not reached by the capillary loops of the spongiosa (Dixon and Perkins in Bourne, 1956) and is thus bathed only in intercellular fluid and is not in contact with the high content of organic phosphate esters in the red blood cell. Thus, the main source of phosphate esters must be derived ultimately from glycogen.

It should be noted that while thalidomide appears to affect the metabolism of cartilage it does not seem to affect the mechanism of ossification. Thus the cartilage model is abnormal but the actual ossification appears normal. Support is provided by the observation, previously noted, that endochondral bones may be abnormal but periosteal membrane bones develop normally. It is not, then, simply a matter of delayed ossification. Endochondral ossification occurs relatively late, but intramembranous ossification occurs earlier and
shortly after the cartilage models are formed.

Alkaline Phosphatase

The intimate association of alkaline phosphatase with ossification, its absence in cartilage before the onset of hypertrophy, its sudden appearance in the nucleus and cytoplasm of cells undergoing hypertrophic changes preparatory to calcification, and its intense activity in the osteoblast, indicates that the enzyme plays a special role in the process of ossification. The precise nature of its role still remains a matter of conjecture, since several theories have been advanced with only partial acceptance of any one of them.

Robison, in 1923, discovered an alkaline phosphatase in hypertrophic cartilage and developing bone, and noted that its site and concentration were correlated with the onset of calcification in these tissues. He suggested that this enzyme hydrolyzes hexosephosphates, yielding orthophosphate, thus adding to the local concentration of phosphate ions with consequent precipitation of the bone salt. As mentioned previously, hexosephosphates are derived from the breakdown of glycogen in the presence of phosphorylase, the former serving (it is believed) as a potential substrate for phosphatase in zones of calcification. A demonstration in this and other investigations of the simultaneous presence of glycogen and phosphatase in the hypertrophic cells tends to support the case.

Another theory has been proposed by Neuman and Neuman (1958), in which they suggest that ester phosphates strongly absorbed by bone mineral act as crystal poisons, inhibiting crystal growth. Phosphatase, by hydrolyzing the ester phosphates, would permit
crystallization to occur on a template which would not otherwise take place in the presence of the ester phosphates.

Recapitulating the findings of this investigation, little or no phosphatase activity was observed in the hypertrophic cells of abnormal sterna, but activity was evident in the osteoblasts of periosteal membrane bone. It is not the purpose of this investigation to assign a role to alkaline phosphatase in calcifying tissues, but rather to point out the implications of its reduced activity in the calcifying cartilage. Whether one subscribes to Robison's original scheme, to a modified version thereof, or to Neuman and Neuman's proposal that alkaline phosphatase acts locally to destroy an organic phosphate believed to inhibit the nucleation of crystals of bone material, the ultimate effect on mineralization namely, retardation, will be observed. Calcification of the cartilagenous matrix is not completely inhibited in the abnormal sterna, but crystal growth is slower as judged by the presence of numerous small centers of bone mineral distributed throughout the centre of ossification.

Fibers

Glycogen

Pritchard (1952) suggested that glycogen may be held in the pre-osseous cells and pre-hypertrophic cartilage cells as a store of potential energy to be used in the intense metabolic processes accompanying cell differentiation and secretion. Bourne (1956) points out that many cells accumulate glycogen before entering into a phase of synthesis, and it may be that the accumulation of glycogen in bone-forming cells is associated with the secretion of the fibrous protein
component of the matrix as well as the secretion of mucopoly-
saccharides, which may serve as an interfibrillar cement (Eastoe in
Bourne, 1956).

In the abnormal sterna studied in this investigation, this
"store of potential energy", glycogen, is locked up so that it is
incapable of normal utilization. Nevertheless, fibers were demonstrated
by staining, which suggests either that fibrillogenesis proceeds
normally or that minor modifications are not demonstratable by the
techniques employed.

Phosphatase

Important implications may be drawn from the reduced alkaline
phosphatase activity, such as was seen in the drug-treated group of
this investigation. That phosphatase is concerned with the produc-
tion and maturation of the protein matrix on which the bone salts are
received has been suggested by a number of authors. Bourne (1943,
1948) has shown that the first formed fibers in bone regeneration
are impregnated with phosphatase and that if the fiber is formed at
all it also contains the enzyme. Many papers describing the
correlation between the formation of protein fibers and the distri-
bution of alkaline phosphatase have been written (see Danielli, 1953;
Bourne, 1956). That alkaline phosphatase is involved in the formation
of the fibrous protein component of the matrix has also been suggest-
ed by Gibson (1957), based on a histochemical and cytological study
of membrane bone development in chicks.

In the present investigation, alkaline phosphatase activity
was significantly reduced in the drug-treated group, but no direct
effect on the protein component of the matrix was observed. That alkaline phosphatase was present in small but perhaps sufficient amounts, tends to explain the observations. Collagen fiber formation appeared to be normal and protein synthesis judged by the cytoplasmic content of ribonucleic acid, appeared normal.

Ribose Nucleic Acid

It has been suggested (Caspersson, 1947) that a great increase in cytoplasmic basophilia (ribonucleic acid) is diagnostic of a cell actively engaged in protein manufacture. Taking this evidence in conjunction with the simultaneous increase in the intercellular matrix, Pritchard (1952) concluded that the osteoblast is actively secreting the protein basis of the matrix. Gibson (1957), investigating the development of membrane bone in the chick, concluded that the cells rich in ribonucleic acid are engaged in the production of protein components of the matrix.

In view of the work of Caspersson (1947), Pritchard (1952) and Gibson (1957) maximum ribonucleic acid content in the chondroblasts and osteoblasts of the developing sternum is probably associated with the elaboration of the fibrous protein component of both the cartilage and bone matrix, respectively. It was further demonstrated, in this investigation, that a decrease in the ribonucleic acid content of chondroblasts and osteoblasts was not observed in the treated group. The results therefore suggest that at least the fibrous protein component of the matrices of cartilage and bone develop normally in the drug-treated group.

Ground Substance
Glycogen

It was suggested earlier that thalidomide or one of its metabolites, may effect a block at the site of phosphorylase activity, resulting in the observations recorded, namely, the presence of large stores of glycogen in disintegrating cells, suggesting an inhibition of glycogen degradation and utilization in the abnormal embryos.

Reference again to figure 2 will reveal that a block at A, resulting in an inhibition in the formation of glucose 1-phosphate from glycogen and inorganic phosphate, will hamper the formation of mucopolysaccharides forming an essential part of the ground substance of cartilage and bone. (A block at B is unlikely since the synthesis of glycogen was not shown to be inhibited.) Pritchard (1952) on the basis of histochemical observations, suggested that in bone and cartilage, the temporary disappearance of glycogen from the osteoblast and chondroblast is associated with the appearance of mucopolysaccharides in the intercellular matrices and postulated that the breakdown of glycogen is associated with the manufacture of the mucopolysaccharides. UDP glucuronic acid, almost certainly an intermediate in the synthesis of chondroitin sulphate and hyaluronic acid (Boström, 1958) is ultimately derived from the breakdown of glycogen into glucose 1-phosphate, suggesting the importance of the phosphorylase mechanism and the implication of its inhibition.

How, then, can we account for the presence of mucopolysaccharides in the ground substance of the treated individuals? Firstly, another source of glucose 1-phosphate exists, which may augment that derived from the breakdown of glycogen. The availability
of glucose and gluconeogenic substances such as amino acids, could serve as a starting point for such a substitute pathway leading to the formation of glucose 1-phosphate and other intermediates necessary for mucopolysaccharide synthesis. Secondly, it was mentioned earlier that the block may be partial so that the synthesis of mucopolysaccharides is obstructed but not completely inhibited.

Preparations of glycogen phosphorylase in muscle have been reported to contain small quantities of pyridoxal phosphate (White, Handler, Smith, Stetten, 1959). Removal of the latter by appropriate procedures causes inactivation of the enzyme, the activity of which is restored upon readdition of pyridoxal phosphate. In view of the similarity between glycogenolysis in muscle and in calcifying cartilage, it seems reasonable to suggest that pyridoxal phosphate is a requisite cofactor of cartilage phosphorylase. Based on this assumption, the phosphorylase block could be effected by (1) inhibiting phosphorylase, (2) competing with pyridoxal phosphate for the active site on the enzyme or (3) interfering with the activation of phosphorylase a step involving a cyclic, high-energy phosphate.

Pyridoxal phosphate is the phosphorylated derivative of pyridoxine, which in turn is a member of the vitamin B complex. Particularly notable among the signs and symptoms of pyridoxine deficiency are polyneuritis and glossitis (White, Handler, Smith and Stetten, 1959), equally notable as side effects of thalidomide. Kuenssberg et al. (1961), as well as a number of others, reported numerous cases of peripheral neuritis after thalidomide administration; Robertson (1962), reported that symptoms of glossitis, developing after thalidomide administration, were cleared up by
Vitamin B complex treatment. These observations suggest an interference with pyridoxine, although a general pyridoxine deficiency need not be the case. Interference may easily be limited to one site of action of a particular enzyme.

Alkaline Phosphatase

Considered in the light of recent investigations concerning the role of alkaline phosphatase in bone development, reduced activity of this enzyme (as demonstrated histochemically) may be reflected in (1) the elaboration of the protein component of the matrix, (2) the mucopolysaccharide component of the matrix and (3) the calcification of the cartilage matrix.

The possible role of alkaline phosphatase in the formation of the mucopolysaccharide component of the matrix has been suggested by Kroon (1952), Gibson (1957) and others. Kroon suggested that the sugars necessary in the formation of mucopolysaccharides were liberated by alkaline phosphatase from the hexosephosphates formed by the phosphorylytic degradation of glycogen. Histochemical evidence presented in this investigation, namely, inhibited degradation of glycogen and reduced phosphatase activity, imply a double insult when interpreted in view of Kroon's suggestion.

PAS Reaction

Staining the ground substance of drug-treated sterna with PAS did not yield any direct evidence that mucopolysaccharide synthesis was inhibited by thalidomide. No measurable difference in staining intensity, between normal and abnormal animals, was observed. However, this result does not rule out the possibility that the ground substance
of abnormal animals may be qualitatively different from normal animals.

Metachromasia

It is accepted that acid mucopolysaccharides exhibit metachromasia with basic aniline dyes such as toluidine blue, demonstrating the presence and degree of polymerization of the mucopolysaccharides. In decalcified sections of cartilage, no measurable difference in staining quality, between normal and abnormal animals, was observed.

Siffert (1951) found that the metachromatic staining of calcified cartilage disappeared as calcification took place. In the present investigation, when the ground substance of the bone (undecalcified sections) of normal animals was stained with toluidine blue, the results agreed with that of Siffert, that is, metachromasia was abolished. The ground substance of drug-treated animals (undecalcified sections) on the other hand, still exhibited metachromasia after toluidine blue staining. The persistence of metachromasia in the ground substance would indicate that there is an interference in the calcification of the cartilagenous core of the newly deposited bone.

Pyronin Y Reaction

Although this investigation did not conclusively demonstrate a difference in staining quality between normal and abnormal animals (that is, with PAS or toluidine blue stain) there is some evidence that there may be a qualitative or quantitative difference in the ground substance. When stained with Pyronin Y, the ground substance of normal animals is stained pink while that of the drug-treated animals is stained an intense reddish-orange.

One explanation may be that the fibers are better visualized
in the drug-treated group because the mucopolysaccharides are less highly polymerized or less plentiful and do not obliterate the fibers to the same extent as in the normal animals.

Earlier in the discussion, it was mentioned that mucopolysaccharides may serve as an interfibrillar cement and the role of glycogen in mucopolysaccharide synthesis and fiber development was discussed. Since there is evidence that the cement is chemically bound to the fibers, abnormal metabolism of the mucopolysaccharides might leave certain protein radicles free to combine with Pyronin Y thus explaining the observations.
SUMMARY AND CONCLUSIONS

1. The development of the sternum was investigated in normal and thalidomide-treated rat embryos, from the time of the first appearance of the sternal primordia through to the end of the gestation period (that is, from 14-23 days of gestation). The histology and histochemistry of the developing sternum of thalidomide-treated and control groups was compared.

2. Particular attention was given to the segmentation of the sternum and especially to the histological and histochemical changes taking place in the sternocostal junctions.

3. The influence of the ribs on the pattern of segmentation was discussed.

4. The distribution of glycogen, alkaline phosphatase, ribonucleic acid and calcium in the developing sternum of normal and treated animals, was studied using histochemical methods.

5. An explanation of the underlying cause(s) of the observed sternal malformations was attempted, based on the histochemical observations recorded.

6. Growth retardation of the ribs and sternum appear to originate through a general mesodermal growth deficiency producing a modification of sternal pattern, indirectly, by way of the ribs.
7. The sternebrae hypertrophy and subsequently ossify in conformity to the pattern of sternocostal articulation.

8. Although movement of the sternal bands does not seem to depend on pressure exerted by the ribs, growth retardation of the latter seems to place a restriction on sternal migration, resulting in bifurcated sterna.

9. Previous authors suggested that the ribs inhibit the hypertrophy and ossification of the cells forming the sternocostal joint. However, the results of this investigation suggest that the potentially ossifying sternal tissue is replaced by the proliferation of non-ossifiable costal cartilage, resulting in the formation of a transverse joint.

10. Abnormal metabolism of the sternal cartilage of drug-treated animals is reflected in its apparent inability to (a) mobilize cytoplasmic stores of glycogen, and (b) elaborate normal levels of alkaline phosphatase. It is postulated that a partial block at the level of phosphorylase may be the cause of the inhibited degradation of glycogen. This would imply (a) an obstruction in the synthesis of the mucopolysaccharide component of the ground substance and (b) retarded calcification of the cartilagenous matrix.

11. Calcification of the cartilagenous matrix, in the drug-treated group, is retarded. Both direct and indirect evidence is presented. However, that matrix which is formed appears to become calcified, normally.
12. The results suggest that the fibrous protein component of the matrices of cartilage and bone develop normally in the drug-treated group. No conclusive evidence was presented regarding the mucopolysaccharide component of the ground substance.

13. Thalidomide affects the metabolism of cartilage but not the mechanism of ossification. Endochondral bone formation is abnormal in the treated group but membrane bones are normal.
FIGURES

Figure 3. Normal 16-day embryo. Showing the close association between the pre-cartilagenous sternal band and the clavicle and ribs of the corresponding side.
Mallory's stain. 84X.

Figure 4. Normal 17-day embryo. Fusion of the sternal bands is almost complete. Note bilateral symmetry of sterno-costal articulations. Aldehyde fuchsin - phloxine - fast green stain. 72X.

Figure 5. Abnormal 17-day embryo. The sternal bands diverge laterally from the level of the fourth rib caudally. Note the bilateral asymmetry of sterno-costal articulations. Aldehyde fuchsin - phloxine - fast green stain. 72X.
Figure 6. Normal 18-day embryo. Fusion of the sternal bands is complete. Note bilateral symmetry of sternocostal articulations. Hematoxylin and eosin stain. 72X.

Figure 7. Abnormal 18-day embryo. Fusion of the sternal bands is still not complete beyond the level of the fourth sternocostal junction. Note bilateral asymmetry of sternocostal articulations associated with some torsion in sternum. Hematoxylin and eosin stain. 72X.

Figure 8. Normal 20-day embryo. Chondroblastic hypertrophy is seen in the intercostal regions of the sternum. Small-celled cartilage persists in the costosternal junctions. Aldehyde fuchsin - phloxine - fast green stain. 120X.

Figure 9. Normal 21-day embryo. The hypertrophic cartilage is invaded by vascular mesenchyme which penetrates the periosteal bone. Mallory's stain. 120X.
Figure 10. Normal 22-day embryo. The different zones of cells can be identified: (1) central core of eroded cartilage, (2) zone of hypertrophy, (3) zone of proliferation and (4) collar of perio-steal bone. Aldehyde fuchsin - phloxine - fast green stain. 120 X.

Figure 11. Normal full term embryo. There are six sternebrae and seven vertebrosternal ribs. Note absence of ossification at sternocostal junction. Alizarin red S stain. 15X.

Figure 12. Abnormal full term embryo. There are only five sternebrae and six vertebrosternal ribs. Alizarin red S stain. 15X.

Figure 13. Abnormal full term embryo. Note shifting of sternocostal articulations and associated fusions of sternebrae. Alizarin red S stain. 15X.
Figure 14. Abnormal full term embryo. Note asymmetry of sternocostal articulations and associated malformation of the sternebrae. Alizarin red S stain. 15X.

Figure 15. Abnormal full term embryo. Note asymmetry of sternal elements as a result of fusions and abnormal spacing of consecutive ribs. The sternebrae are ossified in conformity to the pattern of sternocostal articulation. Alizarin red S stain. 15X.

Figure 16. Abnormal full term embryo. Note dual nature of ossification centers and missing fifth sternebra. Alizarin red S stain. 15X.

Figure 17. Abnormal full term embryo. Note partially bifurcated sternum and missing fifth sternebra. Alizarin red S stain. 15X.
Figure 18. Abnormal full term embryo. Note pronounced bifurcation at anterior end of sternum.
Alizarin red S stain. 15X.

Figure 19. Abnormal full term embryo. The sternum is bifurcated throughout its entire length.
Alizarin red S stain. 15X.

Figure 20. Abnormal full term embryo showing completely bifurcated sternum. Note increased width of sternal bands, reduced length of sternum, crowding of ribs 5-7 and associated loss of fifth sternebra. Alizarin red S stain.
15X.

Figure 21. Abnormal full term embryo displaying an extreme version of the condition described in figure 20. Alizarin red S stain. 15X.
Figure 22. Normal 19-day embryo showing the distribution of glycogen. Note intense glycogen storage in intercostal regions and decreased storage in sternocostal junctions. Periodic acid - Schiff technique. 120X.

Figure 23. Normal 19-day embryo. Higher magnification of figure 22. Periodic acid - Schiff technique. 312X.

Figure 24. Abnormal 19-day embryo, showing the distribution of glycogen in the intercostal regions and sternocostal junctions. Periodic acid - Schiff technique. 120X.

Figure 25. Abnormal 19-day embryo. Higher magnification of figure 24. Periodic acid - Schiff technique. 312X.
Figure 26. Normal 21-day embryo. The central area of hypertrophic cells is completely depleted of glycogen. Periodic acid - Schiff technique. 120X.

Figure 27. Abnormal 21-day embryo. Storage of glycogen is maintained in the hypertrophic cells. Periodic acid - Schiff technique. 312X.

Figure 28. Normal 22-day embryo. The central eroded area of hypertrophic cells is completely depleted of glycogen. At the borders of the zone of erosion, a few cells contain glycogen. Periodic acid - Schiff technique. 120X.

Figure 29. Abnormal 22-day embryo. Heavy glycogen stores persist in the lacunae of disintegrating cartilage cells. Periodic acid - Schiff technique. 120X.
Figure 30. Normal 22-day embryo. Higher magnification of figure 28. Periodic acid - Schiff technique. 312X.

Figure 31. Abnormal 22-day embryo. Higher magnification of figure 29, showing persistence of glycogen in lacunae after disintegration of cartilage cells. Periodic acid - Schiff technique. 312X.

Figure 32. Normal 22-day embryo, showing periosteal bone. Note absence of glycogen from osteocytes. Periodic acid - Schiff technique. 570X.
Figure 33. Normal 23-day embryo. Note almost complete absence of glycogen in hypertrophic cartilage cells bordering the zone of erosion. Spicules of endochondral bone have been laid down in the eroded area. Note the extent of endochondral bone formation and the size and orientation of the spicules. Periodic acid - Schiff technique. 120X.

Figure 34. Abnormal 23-day embryo. Note the intensity of glycogen storage in the hypertrophic cartilage. The bone spicules are of small size and disoriented. Periodic acid - Schiff technique. 120X.

Figure 35. Normal 23-day embryo. Higher magnification of figure 33. Periodic acid - Schiff technique. 312X.

Figure 36. Abnormal 23-day embryo. Higher magnification of figure 34. Periodic acid - Schiff technique. 312X.
Figure 37. Normal 21-day embryo showing the distribution of alkaline phosphatase in a sternebra undergoing ossification. Note the heavy deposition of enzyme in the hypertrophic cells, periosteal osteoblasts and vascular mesenchyme and absence of enzyme from small-celled cartilage of sternocostal junction. Gomori's technique. 120X.

Figure 38. Abnormal 21-day embryo, showing the virtual absence of phosphatase from the hypertrophic cartilage cells and moderate activity in the periosteal osteoblasts. Gomori's technique. 120X.

Figure 39. Normal 21-day embryo, showing intense activity in both nucleus and cytoplasm of hypertrophic cells and periosteal osteoblasts. Note enzyme activity in the intercellular matrix. Gomori's technique. 312X.
Figure 40. Normal 23-day embryo, showing continued enzyme activity in hypertrophic cells, periosteal osteoblasts and vascular mesenchyme. Note trabeculae of bone adjacent to the hypertrophic zone of cartilage. Gomori's technique. 120X.

Figure 41. Abnormal 23-day embryo. Note weak enzyme activity in hypertrophic cartilage and the absence of bone spicules. Enzyme activity in periosteal osteoblasts and vascular mesenchyme is normal. Gomori's technique. 120X.

Figure 42. Abnormal 23-day embryo. Another embryo from the same litter as that pictured in figure 41. Shows absence of phosphatase activity in hypertrophic cartilage but intense activity in periosteal osteoblasts and vascular mesenchyme. Gomori's technique. 120X.
Figure 43. Normal 23-day embryo, showing higher magnification of figure 40. Note intense activity in osteoblasts lining the borders of trebeculae of endochondral bone. Gomori's technique. 312X.

Figure 44. Abnormal 23-day embryo, showing higher magnification of figure 41. Note the weak phosphatase activity of hypertrophic cartilage cells. Gomori's technique. 312X.

Figure 45. Normal 23-day embryo, showing intense phosphatase activity in periosteal osteoblasts and the absence of enzyme from osteocytes. Gomori's technique. 570X.

Figure 46. Abnormal 23-day embryos, showing periosteal bone. Note intense activity in periosteal osteoblasts and diminished activity in osteocytes embedded in the matrix. Gomori's technique. 570X.
Figure 47. Normal 21-day embryo, showing the presence of calcium deposits in the cartilage matrix. Von Kossa's technique. 312X.

Figure 48. Normal 22-day embryo, showing heavy deposits of calcium in the central eroded area. Von Kossa's technique. 120X.

Figure 49. Normal 23-day embryo, showing heavily calcified trebeculae of bone projecting into the eroded area. Note the size and orientation of the trebeculae. Von Kossa's technique. 120X.

Figure 50. Abnormal 23-day embryo, showing decreased size and disorientation of the calcified spicules. Von Kossa's technique. 120X.
Figure 51. Normal 19-day embryo, showing the distribution of cytoplasmic basophilia in the intercostal region and small-celled cartilage of the sternocostal joint. Methyl green and pyronin Y stain. 312X.

Figure 52. Abnormal 19-day embryo. Note the uniform distribution of cytoplasmic basophilia in the mature chondrocytes and small-celled cartilage of the sternocostal joint. Methyl green and pyronin Y stain. 312X.

Figure 53. Normal 20-day embryo. Note the loss of cytoplasmic basophilia in the hypertrophic cartilage cells. Methyl green and pyronin Y stain. 312X.

Figure 54. Abnormal 20-day embryo. Note the intensity of staining in the ground substance of the cartilage. Methyl green and pyronin Y stain. 312X.
Figure 55. Normal 21-day embryo, showing intense cytoplasmic basophilia of periosteal osteoblasts and the loss of activity in the hypertrophic cartilage. Note the lightly staining ground substance. Methyl green and pyronin Y stain. 312X.

Figure 56. Abnormal 21-day embryo, showing intensely stained ground substance. Methyl green and pyronin Y stain. 312X.

Figure 57. Normal 22-day embryo, showing loss of cytoplasmic basophilia in hypertrophic cartilage cells and uniformly stained ground substance. Methyl green and pyronin Y stain. 312X.

Figure 58. Abnormal 22-day embryo, showing loss of cytoplasmic basophilia in hypertrophic cartilage cells and irregularly stained ground substance. Methyl green and pyronin Y stain. 312X.
Figure 59. Normal 23-day embryo, showing osteoblasts lining large spicules of bone. Methyl green and pyronin Y stain. 312X.

Figure 60. Abnormal 23-day embryo, showing small, disoriented bone spicules. Methyl green and pyronin Y stain. 312X.
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DEVELOPMENT OF THE STERNUM IN
THALIDOMIDE-TREATED RATS

by

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A HISTOLOGICAL AND HISTOCHEMICAL STUDY
OF THE DEVELOPMENT OF THE STERNUM
IN THALIDOMIDE-TREATED RATS

An Abstract

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