The Effect of Hydrocortisone on Extracellular Connective Tissue Fibrils in the Early Chick Embryo [1]

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ABSTRACT Two series of chick embryos were treated with hydrocortisone in an attempt to demonstrate the influence of this hormone upon microfibrils found free in the tissue space surrounding the notochord. Embryos in series I were explanted on agar-albumen medium and permitted to survive 1–24 hours after Ringer's solution or hydrocortisone had been pipetted onto the area vasculosa. Doses of hydrocortisone ranged from 5 μg to 1 mg. Series II embryos were similarly treated *in ovo* through a small shell "window" and then incubated 12 or 24 hours. All embryos were sacrificed at 72 hours incubation age. Ringer-treated embryos in both series exhibited a rich tangle of early extracellular connective tissue fibrils (microfibrils) surrounding the notochord. One hour after treatment with hydrocortisone, embryos in series I showed a reduction in perinotochordal microfibrils. At four hours the effect was maximal and by 12 hours, recovery had been initiated, the morphology of which suggested a re-organization of amorphous material and extracellular debris to form fibrillar structures. Twenty-four hours after treatment, recovery was complete and hydrocortisone-treated embryos exhibited perinotochordal fibril populations that were indistinguishable from Ringer-treated specimens. The results of the series II experiments roughly paralleled those seen in series I but were somewhat less predictable.

Possible modes of action of hydrocortisone on connective tissues and their relationship to the present study are discussed. It is suggested that the steroid may induce the release of a substance with an enzymatic activity capable of digesting microfibrils. This catabolic activity may be reflected as an inhibition of connective tissue production if it is assumed that microfibrils are precursors of larger, more mature fibrils. It is further suggested that since microfibrils are thought to contain connective tissue proteins, the initiation of microfibrillar reduction by hydrocortisone could indicate that this hormone may act in a similar manner on more mature connective tissue fibrils.

Intensive research has been carried out to determine the basic mechanism underlying the action of corticosteroids on the connective tissues. This has been the outcome of early observations on inhibition of wound healing in patients being treated with cortisone (Ragan et al., '50; Cole et al., '53). The history of these investigations has been adequately reviewed (Chen and Postlethwait, '64; Wegelius and Kock, '66; Dougherty and Berliner, '68).

Electron microscopic investigations of the effect of corticosteroids on the connective tissues are few. Asadi et al. ('56) showed an increase in the aggregation of polymers of mucopolysaccharides in subcutaneous loose connective tissues of hydrocortisone-treated mice. In an investigation designed secondarily to test the effect of hydrocortisone on the synthesis of collagen *in vitro*, Castor and Muirden ('64) demonstrated progressive cytological changes in fibroblasts by electron microscopy. Light micrographs were used, however, to support their belief that hydrocortisone re-

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duced stainable extracellular fibrous material in the tissue cultures. The literature offers no accounts of electron microscopic observations of the influence of hydrocortisone on developing extracellular connective tissue fibrils.

Therefore, it seemed worthwhile to examine the effects of hydrocortisone in a region of the body containing extracellular connective tissue fibrils in various stages of development. The perinotochordal tissue space of the chick embryo at 72 hours, with its well developed boundary (basement) membrane and rich tangle of microfibrils (Low, '68) appeared to meet these requirements.

MATERIALS AND METHODS

Chick embryos of the White Leghorn strain were used in two series. Series I consisted of 31 Ringer-treated and 109 hydrocortisone-treated embryos, while series II included 26 and 54 embryos, respectively. Control embryos in both series were treated with 0.1 ml of Ringer's solution only. All embryos in series I were explanted at 48–71 hours of incubation onto an agar-albumen medium (Spratt, '47). The original technique was modified to include Ringer's solution buffered (Spratt, '49) at pH 8.0. Individual doses of 5, 100, or 500 μg of hydrocortisone-21-phosphate disodium salt[3] were dissolved in 0.1 ml buffered Ringer's solution. A single dose was pipetted onto the area vasculosa of the hormone-treated embryos. These procedures and the duration of the experiments were so timed that the incubation age at sacrifice was 72 hours. Therefore, the developmental stage (Hamilton, '52) of all embryos at the time of sacrifice was nearly constant.

All embryos in series II were treated *in ovo*. Pilot experiments revealed the necessity of increased quantities of hydrocortisone to achieve results similar to those seen in series I. Accordingly, 1 mg doses of hydrocortisone were dissolved in 0.1 ml buffered Ringer's solution and pipetted onto the vitelline membrane through a small shell "window." Experiments in series II were carried out in embryos of 48 or 60 hours incubation age. All embryos in this series were re-incubated for time intervals necessary to bring their final incubation age to 72 hours.

All embryos were sacrificed by immersion *in toto* in Karnovsky's fixative (Karnovsky, '65). The cervical region was excised early during fixation and the resultant tissue block (ca. 2 mm long) was postfixed in OsO_4 (Karnovsky, '65), dehydrated and embedded in Epon (Luft, '61). The cephalic end of each block was oriented for cross-sectional microtomy. By this technique the cephalocaudal position of the areas studied was kept constant. Sections 1 μ thick were stained with toluidine blue for orientation and determination of notochordal position. Thin sections were stained with lead citrate (Venable and Coggeshall, '65) and uranyl acetate (5% in absolute ethanol). These preparations were examined in a Philips EM-200 electron microscope.

OBSERVATIONS

Chick embryos at 72 hours (30–36 somites) possess an established vitelline circulation (fig. 1). A cylindrical notochord exhibiting conspicuous vacuoles, which may occupy up to about 50% of its cross-sectional area, is present (fig. 2). It is surrounded by a nearly acellular zone and is separated dorsally from the neural tube by intervening extracellular space. Secondary mesenchymal cells from the sclerotomes invade the perinotochordal region to become chondroblasts from which vertebral elements originate. However, cells and extracellular materials do not appear immediately adjacent to the notochord in light microscopic preparations of embryos after 72 hours of incubation (fig. 2).

Electron micrographs reveal in chick embryos of 72 hours incubation age a rich tangle of microfibrils entirely surrounding the notochord. Fibrils are concentrated adjacent to the notochordal boundary membrane with the fibrillar component of which they are in intimate association. Small bits of amorphous material may be seen closely associated with the fibrils or in aggregate (fig. 3); they resemble interstitial bodies (Low, '70).

Ringer-treated embryos explanted on albumen extracts (series I) show no grossly observable abnormalities. Circulation is maintained and development proceeds normally but at a somewhat reduced rate. The

[3] Supplied by Merck, Sharpe and Dohme Co., Inc.

perinotochordal regions of Ringer-treated embryos (figs. 4, 6, 10, 12) exhibit an intense concentration of microfibrils in various stages of growth and development. This microfibrillar population remains remarkably constant and is not visibly altered during the explantation period. Ringer-treated specimens in series II (*in ovo*) occasionally exhibit bizarre and exaggerated shapes. However, their perinotochordal fibrillar populations appear normal (figs. 14, 16).

One hour after exposure to hydrocortisone (fig. 5) embryos in series I show a reduction in free extracellular connective tissue fibrils in the perinotochordal space. Loss of the larger microfibrils is particularly striking and the depletion of both fibrils and amorphous material is especially severe in the region nearest the boundary membrane. In these one-hour specimens, foldings and invaginations are occasionally present on the free surface of notochordal cells (fig. 5), some of which contain fibrillar or amorphous material. Comparable areas are noted in Ringer-treated specimens of the same exposure time (fig. 4).

Four hours after treatment with various doses of hydrocortisone, embryos in this series (figs. 7–9) exhibit a maximal reduction of fibrils in the perinotochordal area. Few discernible microfibrils remain in the extracellular space. The area nearest the boundary membrane seems to be affected most severely and the membrane itself appears to have lost some of its fibrillar component.

At 12 hours post-treatment embryos exposed to hydrocortisone (fig. 11) show fewer fibrils than controls (fig. 10) but more than embryos similarly treated for one and four hours. A few large microfibrils associated with bits of amorphous material are present in the extracellular space. Likewise, the fibrillar component of the boundary membrane appears to have increased over that surrounding the notochord in embryos exposed to hydrocortisone for shorter periods.

Twenty-four hours after treatment with hydrocortisone a marked reappearance of extracellular connective tissue fibrils is noted in the perinotochordal space (fig. 13). These microfibrils are located near the subjacent boundary membrane and are of the larger, more mature type. Ringer-treated (fig. 12) and hormone-treated embryos at 24 hours post-treatment are essentially indistinguishable.

Results in series II generally paralleled those seen in the explants but were somewhat irregular. Embryos sacrificed 12 hours after treatment with hydrocortisone demonstrated a severe reduction in extracellular material (fig. 15). In embryos similarly treated 24 hours prior to sacrifice, however, the fibrillar populations (fig. 17) were normal. These embryos showed substantial numbers of large perinotochordal microfibrils.

DISCUSSION

The effects of adrenal corticosteroids on living systems has been exhaustively investigated since the demonstration by Hench and associates ('49) of the ameliorative effects of cortisone and corticotropin on the symptoms of rheumatoid arthritis. These studies have demonstrated that corticosteroids in large "pharmacological" doses exert a profound influence on carbohydrate, lipid and protein metabolism (Williams, '68). Among the effects of hydrocortisone on protein metabolism (and hence connective tissue metabolism) are an increase in protein breakdown and mobilization of amino acids from skin, muscle and bone (Brown, '62; Thomas, '68). These observations emphasize the catabolic action of the steroid. A simultaneous depression in synthesis of collagen, mucopolysaccharides and proteins in general (Ebert and Prockop, '63,'67) indicates the anti-anabolic influence of hydrocortisone and reveals the complexity of the action of this hormone on proteins. Whether the primary effect of hydrocortisone on protein metabolism is catabolic or anti-anabolic is in controversy (Brown, '62).

Recently, O'Malley et al. ('69) have shown that steroid hormones affect their target tissues by combining with specific nuclear receptors. Thus, they may be capable of modifying gene transcription, RNA formation and ultimately protein synthesis (Catt, '70). This concept of steroid action is compatible with a proposed mechanism (Houck and Patel, '65) by which corticosteroids may affect the connective tissues. On the basis of biochemical evidence these workers postulated that hydrocortisone may

potentiate the release of a cellular protease into the extracellular compartment. Their results further indicated that hydrocortisone may be responsible for the induction of collagenolytic activity in the dermis of young rats.

Further support for this mechanism of steroid action is found in enzyme digestion studies of extracellular connective tissue fibrils in the early chick embryo (Carlson and Frederickson, '70). These studies indicate that trypsin, collagenase and hyaluronidase are effective agents in the digestion of perinotochordal microfibrils in chick embryos of 72 and 96 hours incubation age. This could indicate a similarity of the ultimate modes of chemical action of steroids and enzymes on early connective tissue fibrils.

In the present investigation Ringer-treated embryos, explanted on an agar-albumen medium, demonstrated normal perinotochordal microfibrillar populations. Similarly, embryos treated *in ovo* with Ringer's solution only failed to reveal any recognizable ultrastructural alterations in the free microfibrils surrounding the notochord. These observations suggested that neither the explanting process nor the application of Ringer's solution were directly responsible for microfibrillar changes.

Hydrocortisone-treated embryos in series I showed that morphological effects of this hormone can be observed within one hour post-treatment which suggested that hydrocortisone may rapidly induce an "enzyme-like" effect on early extracellular connective tissue fibrils. The effect reached a maximum at about four hours, after which a gradual remission was manifested by a return of the fibril population to a level indistinguishable from that of the controls. This recovery was not unexpected since it is known that corticosteroids are catabolized rapidly by the body (Dougherty and Berliner, '68).

It seemed significant that throughout the experiments of series I, hydrocortisone appeared to exert its effect in a manner that was independent of the dosage employed. Specimens receiving the highest doses (up to 500 μg) demonstrated a capacity for recovery that was not noticeably less effective than that seen in embryos treated with only 5 μg. It was noted further that the area of tissue space nearest the notochordal boundary membrane was the most severely affected by the hormone and was also the last area to recover its normal fibrillar population.

The results of the experiments of series II roughly parallelled those seen in series I. These confirmed the validity of the latter and implied that the explantation technique could not be implicated in causing the fine structural changes observed.

These observations collectively suggested that microfibrils may possess a previously unrecognized sensitivity to the action of hydrocortisone. Furthermore, there was evidence to suggest a chemical or developmental disparity between fibrils nearest the notochord and those located more peripherally.

The reduction in perinotochordal microfibrils seen in the present investigation is consistent with the mechanism of steroid hormone action proposed by Houck and Patel ('65). It seems reasonable that a cellular protease, produced by a hydrocortisone-modified gene, could be operable here. This strongly suggests a catabolic mechanism of action of hydrocortisone on early extracellular connective tissue fibrils. However, it does not preclude the well-documented belief that a primary effect of hydrocortisone on the connective tissues is anti-anabolic (Dougherty and Berliner, '68). For, if it is assumed that microfibrils are a precursor of more mature connective tissue fibers (Anderson, '67) the degrading action of hydrocortisone on microfibrils may ultimately reduce the total production of connective tissue and, hence, be considered anti-anabolic.

Conversely, one may assume that microfibrils contain, at least in part, collagen or other connective tissue proteins (Ross and Bornstein, '69). Then the capacity of hydrocortisone to initiate a reduction in microfibrillar populations could suggest that this steroid may be capable of initiating a similar action on well-developed collagen fibers. Thus, the total effect could be catabolic.

It is possible that neither explanation satisfies all conditions. Perhaps both catabolic and anti-anabolic mechanisms are operable and these may act alternately, simultaneously or both. Whatever may be the mode of action, it is clear from the present

study that hydrocortisone exerts a marked effect on the early growth of connective tissue fibrils in the chick. Since these fibrils are believed to be the forerunners of more mature connective tissues and the locus of study occurs at a stage of development when vertebrate embryos are essentially alike, the fundamental nature of these results should provide a basis for further experimentation.

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Abbreviations

A, aorta	N, notochord
BM, basement membrane	NT, neural tube
I, cellular invagination	TSP, tissue space

PLATE 1

EXPLANATION OF FIGURES

1 Living chick embryo after 72 hours of incubation and explanted onto an agar-albumen medium. Photograph was made with transmitted light. Vitelline circulation and the area vasculosa are intact. Lines indicate position of tissue block removed for study. × 3.

2 Light micrograph of a cross-section taken from the cephalic end of the tissue block indicated in figure 1. The cylindrical notochord (N) is separated dorsally from the neural tube (NT) and ventrally from the aorta (A) by an intervening tissue space (TSP). Secondary mesenchymal cells occupy the tissue space but the area adjacent to the notochord (arrows) is clear. Epon, toluidine blue. × 280.

3 Low-power electron micrograph of the edge of the notochord from a chick embryo of 72 hours incubation age. Extracellular connective tissue fibrils and associated amorphous substances entirely surround the notochord. The most intense concentration of extracellular materials is seen immediately adjacent to the notochordal surface. Occasionally aggregates of amorphous material resembling interstitial bodies (arrow) are noted in the tissue space. × 6,100.


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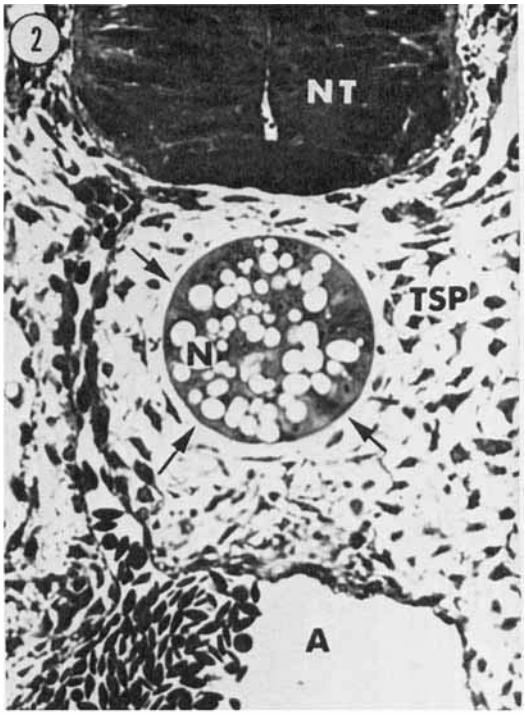
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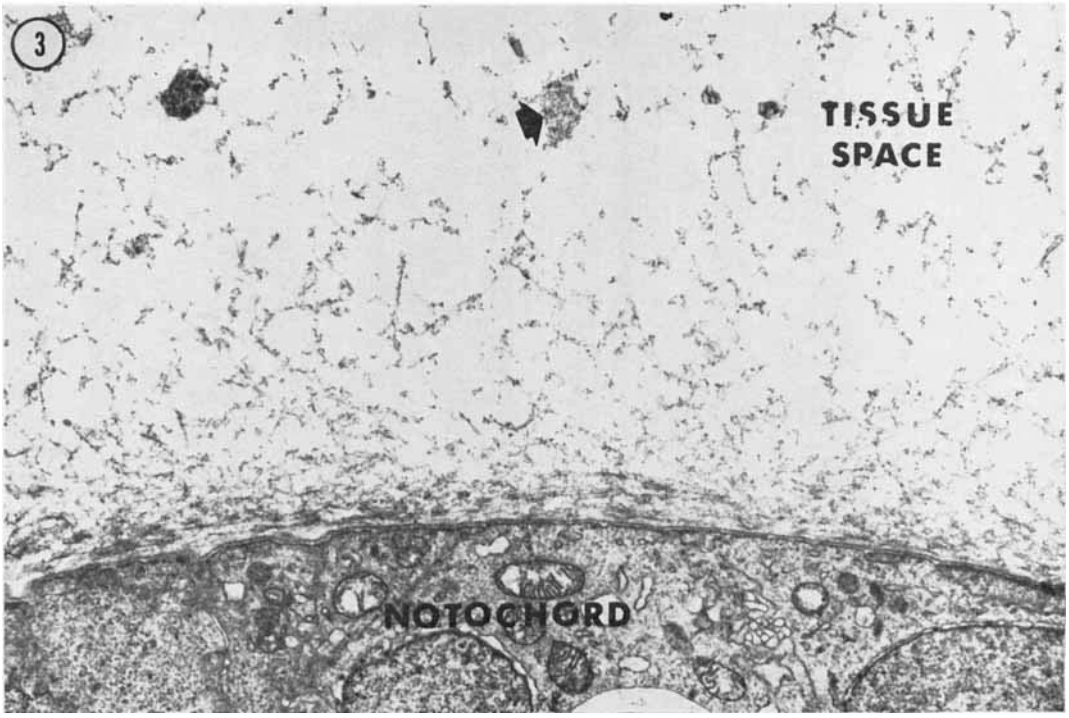
3
TISSUE
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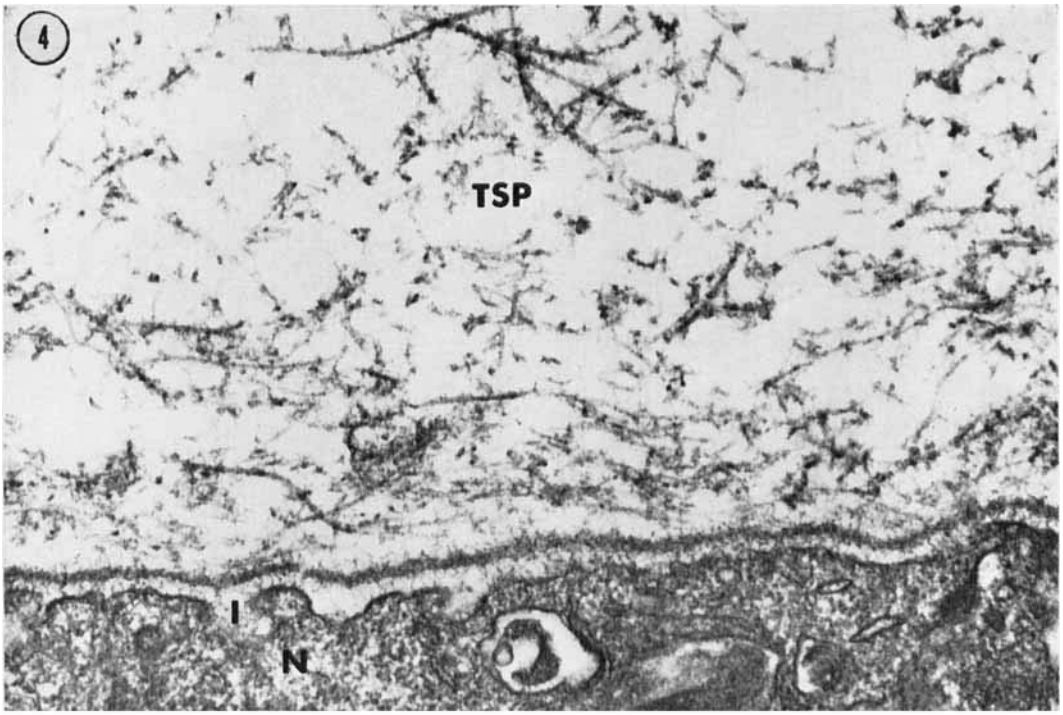
PLATE 2

EXPLANATION OF FIGURES

Explanted embryos (series I) one hour after treatment. Both figures illustrate the edge of the notochord (N) and surrounding tissue space (TSP) in chick embryos of 72 hours incubation age. I, cellular invagination.

4 Embryo treated with 0.1 ml buffered Ringer's solution only. The perinotochordal fibrillar population is normal. $\times$ 36,000.

5 Embryo treated with 100 μg hydrocortisone in 0.1 ml buffered Ringer's solution. Note the loss of microfibrils near the notochordal boundary membrane. $\times$ 33,000.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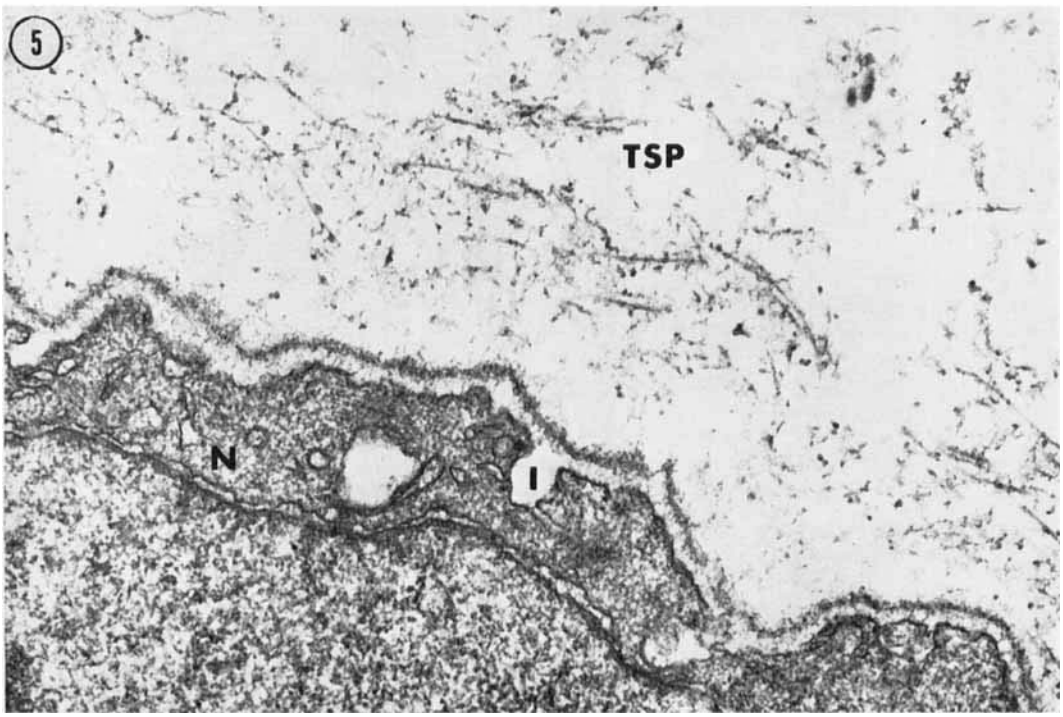
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PLATE 3

EXPLANATION OF FIGURES

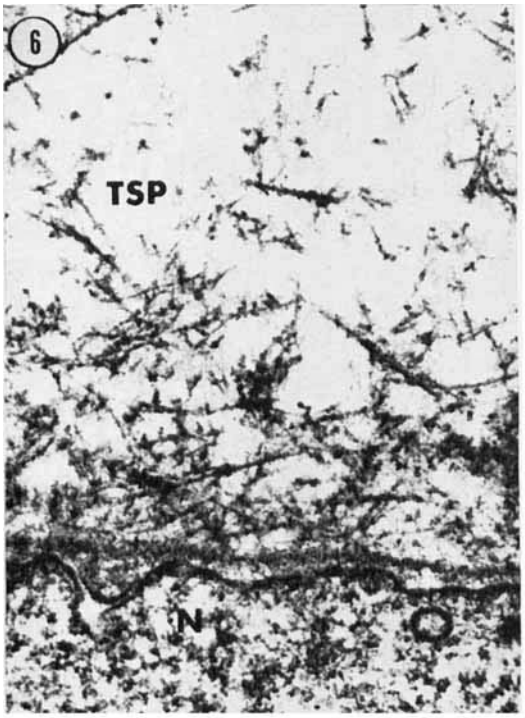
Explanted embryos (series I) four hours after treatment. All figures illustrate the edge of the notochord (N) and surrounding tissue space (TSP). The fibrillar population seen in figure 6 is normal. Figures 7–9 demonstrate the similar effect of different dosages of hydrocortisone on the perinotochordal fibril population. These figures illustrate the maximal degrading effect of the steroid on the extracellular connective tissue fibrils surrounding the notochord. Few discernible microfibrils remain and amorphous material and extracellular debris are the primary occupants of the tissue space.

6 Embryo after treatment with 0.1 ml buffered Ringer's solution only. $\times 33{,}000$.

7 Embryo after treatment with 5 μg hydrocortisone in 0.1 ml buffered Ringer's solution. $\times 34{,}000$.

8 Embryo after treatment with 100 μg hydrocortisone in 0.1 ml buffered Ringer's solution. $\times 36{,}000$.

9 Embryo after treatment with 500 μg hydrocortisone in 0.1 ml buffered Ringer's solution. $\times$ 36,000.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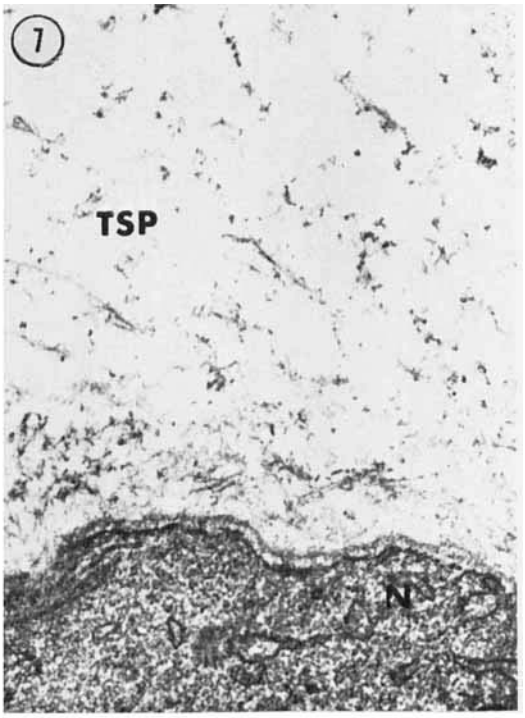
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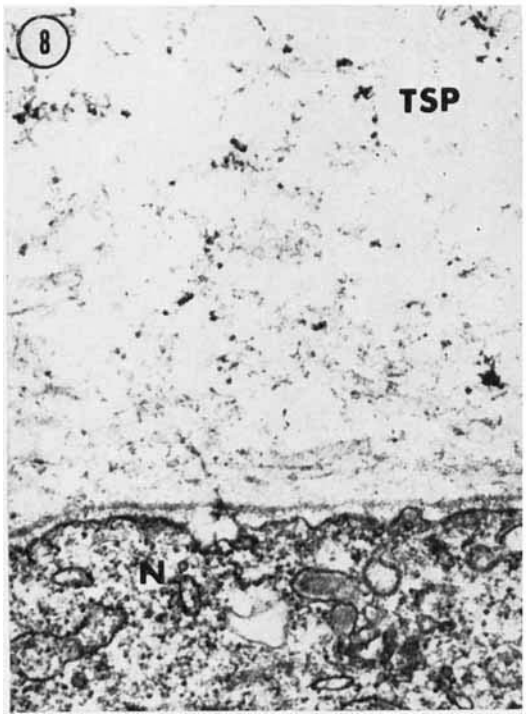
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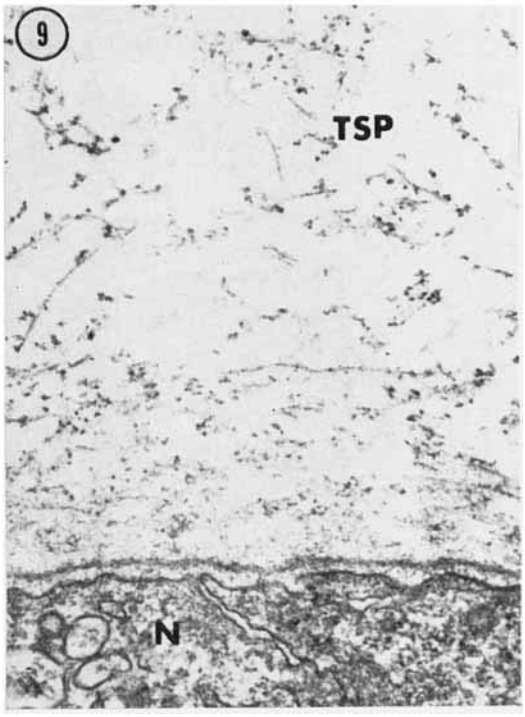
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PLATE 4

EXPLANATION OF FIGURES

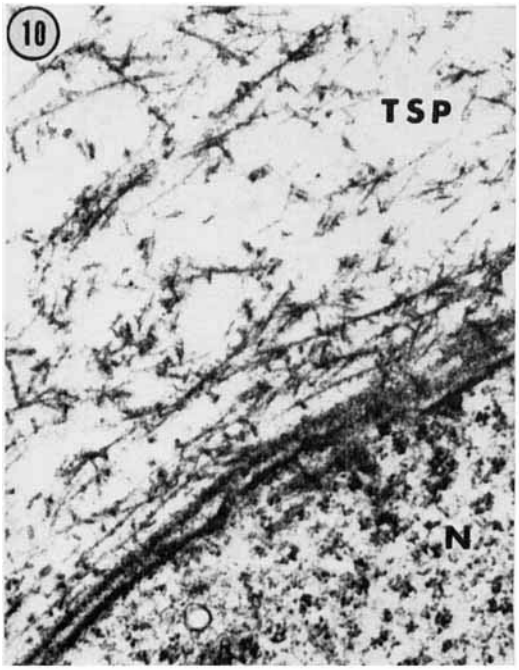
Explanted embryos (series I) 12 or 24 hours after treatment. All figures illustrate the edge of the notochord (N) and surrounding tissue space (TSP).

10 Embryo 12 hours after treatment with 0.1 ml buffered Ringer's solution only. The fibrillar population surrounding the notochord is normal. × 30,000.

11 Embryo 12 hours after treatment with 100 μg hydrocortisone in 0.1 ml buffered Ringer's solution. A re-organization of amorphous material to form recognizable microfibrils (arrows) indicates a partial recovery from the effects of the hormone. × 29,000.

12 Embryo 24 hours after treatment with 0.1 ml buffered Ringer's solution only. The fibril population surrounding the notochord is normal. × 28,000.

13 Embryo 24 hours after treatment with 100 μg hydrocortisone in 0.1 ml buffered Ringer's solution. A normal perinotochordal fibril population indicates full recovery from the effects of hydrocortisone on these early connective tissue fibrils. × 26,000.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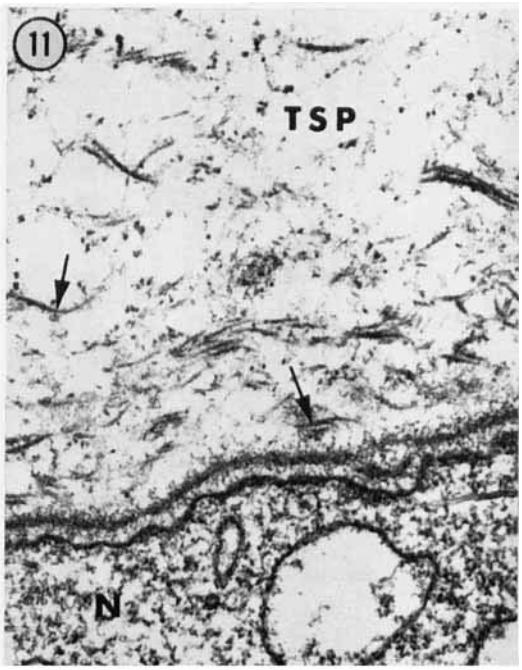
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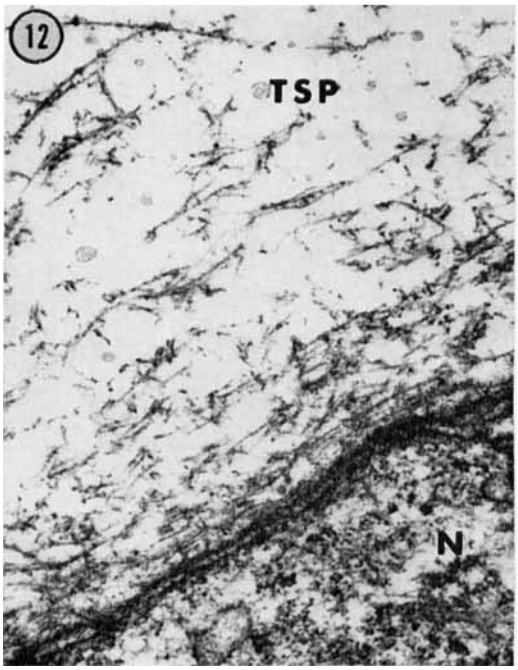
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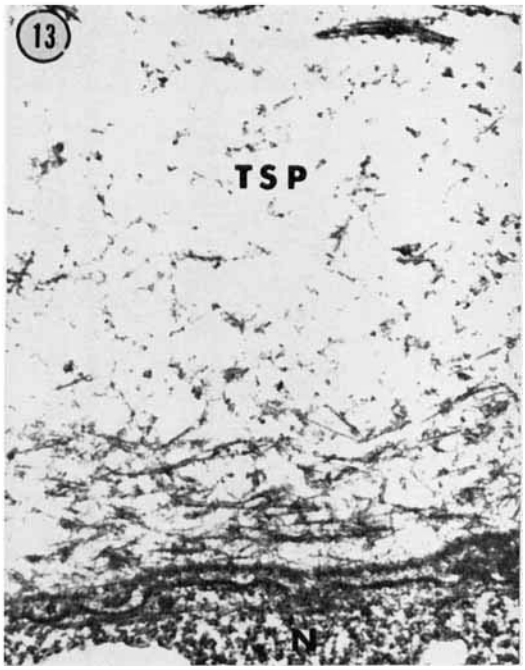
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PLATE 5

EXPLANATION OF FIGURES

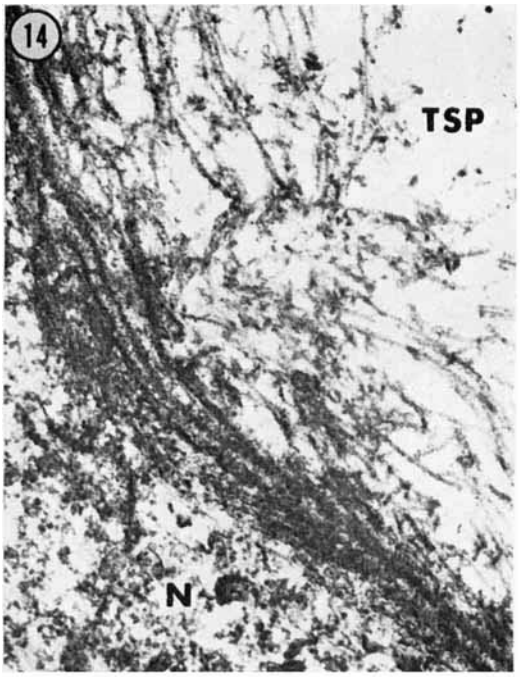
Embryos 12 or 24 hours after treatment *in ovo* (series II). All figures illustrate the edge of the notochord (N) and surrounding tissue space (TSP).

14 Embryo 12 hours after treatment with 0.1 ml buffered Ringer's solution only. The microfibrillar population is normal. ×44,000.

15 Embryo 12 hours after treatment with 1 mg hydrocortisone in 0.1 ml buffered Ringer's solution. Note the loss of fibrillar material. ×45,000.

16 Embryo 24 hours after treatment with 0.1 ml buffered Ringer's solution only. The microfibrillar population is normal. × 34,000.

17 Embryo 24 hours after treatment with 1 mg hydrocortisone in 0.1 ml buffered Ringer's solution. The normal microfibrillar population indicates a recovery from the effects of the hormone. This parallels the recovery seen in explanted embryos (plate 4). ×30,000.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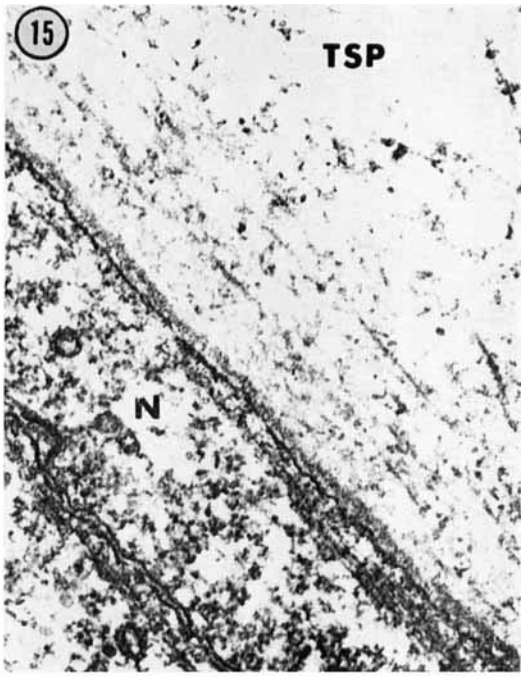
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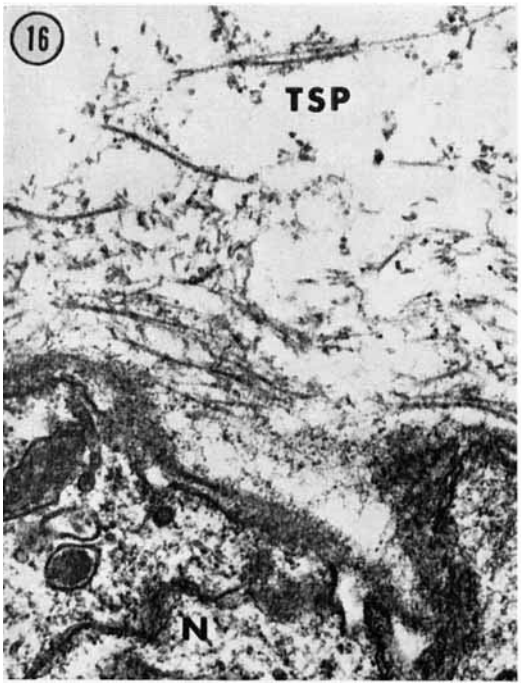
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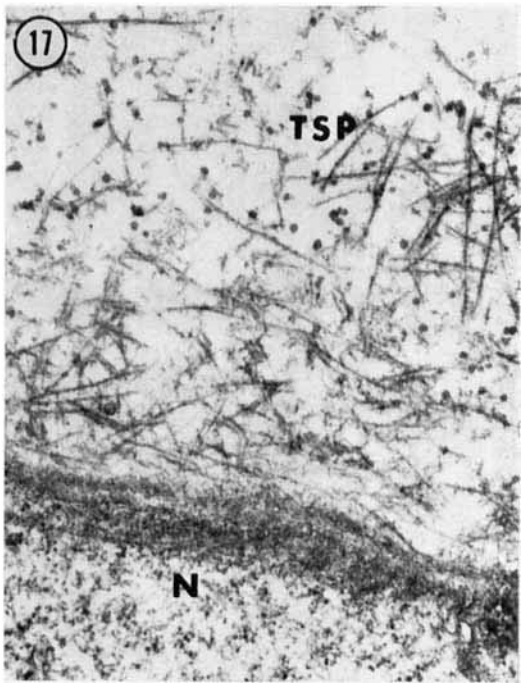
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