OBSERVATIONS ON THE HISTOCHEMISTRY AND FINE STRUCTURE OF THE NOTOCHORD IN RABBIT EMBRYOS

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INTRODUCTION

Although the main facts of the development and histology of the notochord are well known, there appears to be a sparseness of data concerning its histochemistry and fine structure. The notochord has been studied in a variety of animal species but the workers concerned have confined themselves in general to the use of standard staining procedures.

The axial rod of cells which makes up the notochord during the period when the somites are laid down becomes modified as the primordia of the vertebral centra and intervertebral discs are established. Carlier (1890) described the formation of curious V-shaped bends of the rod, the point of the V directed posteriorly, at the sites of the future intervertebral discs in early sheep embryos. He quoted Balfour (1876) and Köllicker (1882) as having noted the same phenomenon. In a sheep foetus of 17·5 mm. C.R. length, Carlier further observed the absence of notochordal cells in the developing centra, but the presence here of a jelly-like matrix which he thought was produced by the perichordal cells. Similar findings were made by Minot (1906) and Williams (1908). The latter, in detailing the histology of the notochord, described it (chiefly in the pig embryo) as first composed of epithelial cells, then turning into a syncytial mucoid connective tissue, and finally becoming 'cellular' again and looking rather like cartilage. He also observed, in one stage of a rabbit embryo, vacuolation of the notochord cells; the notochord sheath is, according to Williams, in the early stages striated concentrically but later develops an inner zone composed of a mucin-like material, staining with mucicarmine, derived from the vacuoles of the notochord cells. Dawes (1930) traced the development of the notochord in the mouse and confirmed the observations of Williams (1908) that it is in turn 'cellular', 'syncytial' and finally 'cellular'. Sensenig (1943) described, in the deer mouse, migration of sclerotomic cells through the notochord sheath and concluded that these cells thus added to the bulk of the nuclei pulposi. The same author (1949), working with human material, found that the development of the sclerotomic perichordal tube coincided with the appearance of a homogenous 'eosinophil elastica externa' and suggested a sclerotomic rather than notochordal origin for this sheath. Peacock (1951, 1952) described the perinotochordal tissue in human embryos as specialized embryonic cartilage, and observed that the notochord cells underwent mucoid degeneration to form the nuclei pulposi. He also found the notochord sheath to stain lightly with basic dyes and to show longitudinal striations. Finally, Duncan (1957), in a study of the early chick embryo with the electron microscope, unfortunately only available as yet in the form of an abstract,

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states that the notochord is surrounded by a halo of ultrafine fibrils. They are 'limited to the vicinity of the notochord' and appear to be 'produced at the surface of the notochord rather than within this structure'.

In view of the relative paucity of information on the actual cytology of the notochord and the structure of its sheath, it was decided to investigate it both histochemically and by means of the electron microscope. The present observations concern only rabbit embryos and are confined to the stages in which the notochord is fully established and undergoing changes associated with the production of the definitive nuclei pulposi.

MATERIAL AND METHODS

Embryos were obtained from does of 13, 15 and 17 days after observed matings and the stages of development of the embryos checked with the table of Minot & Taylor (1905). For light microscopy, embryos were fixed whole in alcoholic Bouin, cold absolute ethyl alcohol or cold absolute acetone. They were dehydrated through graded alcohols and embedded in paraffin wax in the usual manner. Sections were cut at 7 μ, at least one embryo of each stage being cut transversely and one sagittally. The following staining procedures were used: haematoxylin and eosin, Masson's trichrome, Azan, iron haematoxylin–van Gieson, Best's carmine, mucicarmine, alcian blue, Weigert's elastin, Wilder–Gomori silver impregnation for reticulin, the P.A.S. reaction and both the calcium cobalt and azo-dye techniques for alkaline phosphatase. With the material for electron microscopy, the problem of the preparation of specimen blocks prior to fixation arose. It is possible to dissect out the notochord or, at least, the developing vertebral column of embryos at the stages studied but, by doing so, normal relationships and the structure of the notochord may be altered. It was decided to prepare specimen blocks by removing the head and ventral and lateral portions of the thorax and abdomen of each embryo and then cut transverse sections of the remaining dorsal regions about 0·5–1·0 mm. thick. These blocks of tissue, measuring 0·5–1·5 mm.^{3}, were fixed by immersion in 1% osmium tetroxide, which previous experience had shown to penetrate into embryonic tissue more rapidly than into adult tissue. The osmium tetroxide was buffered to pH 7·2 either with acetate veronal (Palade, 1952) or bichromate (Dalton, 1955) and fixation performed at room temperature for periods of either 45 or 60 min. Washing, dehydration, infiltration with and embedding in 5% methyl in *n*-butyl methacrylate were routine, polymerization being effected at 60° C for 24 hr., at which temperature polymerization damage is minimal (Borysko, 1956). Ultrathin sections were cut on a Sims–Leeson heat-advance ultramicrotome (1957), using glass knives, collected on carbon-coated grids and examined in a Metropolitan–Vickers E.M. 3 electron microscope at 75 kV.

RESULTS

Thirteen-day stage

The embryos studied varied from 8·5 to 11·1 mm. C.R. length before fixation. In cross-section, the general shape of the notochord is circular and in a longitudinal section there is no evidence of segmentation, i.e. there is no intervertebral enlargement and no intravertebral constriction. In a 7 μ transverse section (Pl. 1, fig. 1) there are approximately 12–14 markedly chromatic nuclei which are large, circular,

or nearly so. In a longitudinal section (Pl. 1, fig. 4: an electron micrograph), they appear oval in a transverse direction indicating a biconvex disc shape. The cells are arranged in a pattern which is strikingly epithelial in character. Cell boundaries are difficult to define on light microscopy (Pl. 1, fig. 1) but are clearly seen in the low-power electron micrograph of Pl. 1, fig. 4. Small vacuoles are present here and there in the cytoplasm. The notochord sheath is well marked, appears homogeneous and undulates over the contours of the notochord cells. It is eosinophil and stains red with van Gieson, intensely green with Masson's trichrome, blue with azan stain and with alcian blue, and red with mucicarmine. It shows a positive P.A.S. reaction. With Weigert's elastin, some of the more peripheral parts of the sheath stain in places pale purple. Neither in the sheath nor in the cells is there any alkaline phosphatase activity. A condensation of mesenchymal cells around the sheath of the notochord forms the perichordal tube. These cells are arranged concentrically and the nuclei are oval in shape. The notochord is avascular in this and all other stages examined.

In high-power electron micrographs, the nuclear membrane is double and the notochord cells show clearly defined cell membranes with no intercellular material (Pl. 1, fig. 5). Mitochondria and endoplasmic reticulum are sparse. β-cytomembranes are not seen. The notochord sheath contains microfibrils which are cut mainly transversely in a cross section and often appear continuous with the basal layer of the cell membrane of the notochord cells. There is no outer lining membrane to this sheath and the microfibrils do not penetrate between the mesenchymal cells of the perichordal tube.

Fifteen-day stage

The embryos studied varied from 14·9 to 15·9 mm. C.R. length before fixation. The mesenchyme of the vertebral column now shows condensations of cells in the regions of the developing intervertebral discs (Pl. 1, fig. 2). The notochord also begins to show evidence of segmentation, its cells being more numerous in the intervertebral regions than in the sites of the developing centra. The cells are still arranged in a close epithelial pattern and there is no sign of a network formation. They contain numerous glycogen granules and droplets. Beginning segmentation is also evident in the sheath (Pl. 1, fig. 2). Although its over-all diameter is fairly uniform, the clearly staining part thins out in the regions of the centra and is here separated from the notochord cells by an interval which takes up little stain, and may even appear clear, probably due partially to shrinkage. The staining properties of the sheath are similar to those observed in the earlier stage except that the red stain with van Gieson is confined to the most peripheral parts of the sheath. Irregular purple staining with Weigert's elastin is still evident. There is no staining with the Wilder–Gomori silver impregnation. The P.A.S. reaction is also more intense in the very periphery and persists after pre-treatment of the section with saliva. Staining with Best's carmine confirms the absence of glycogen in the sheath; there is no alkaline phosphatase activity.

Examination of the notochord cells with the electron microscope shows little difference from the 13-day stage and only slight variations between the intervertebral and intravertebral regions. The main change is an increase in the degree

of vacuolization of the cells (Pl. 2, fig. 6) which, at least in part, is due to their glycogen contents. Cell membranes are again well defined (Pl. 2, figs. 6, 7). The basal cell membranes show spike-like projections into the inner part of the sheath in the intravertebral regions where two clearly defined zones can be distinguished, an inner zone of less density and an outer dense zone with irregularly arranged microfibrils (Pl. 2, figs. 6, 8). The latter corresponds to the deeply stainable part as seen in light microscopy, whilst the inner zone seems to correspond, at least in parts, to the pale interval mentioned above. In the intervertebral regions the notochord cells are associated closely with the microfibrils of the sheath, although even here there is, in places, some greater aggregation of microfibrils at the periphery (Pl. 2, fig. 7). The direction of the fibrils varies with the regions, being longitudinal in the intervertebral regions and predominantly circular in the intravertebral regions. There is no limiting membrane externally and the mesenchymal cells of the perichordal tube are closely associated with the outer part of the sheath.

Seventeen-day stage

The embryos studied varied from 18·0 to 21·2 mm. C.R. length before fixation. The notochord now shows intervertebral dilatations and intravertebral constrictions (Pl. 1, fig. 3), except in the basisphenoid and coccygeal regions where it retains its primitive cylindrical form. In the dilatations and their extensions into the intravertebral regions, there is a high degree of vacuolization in or/and between the notochord cells which gives to the whole an appearance somewhat like a 'reticulated epithelium'. Cell boundaries cannot be discerned and the impression is gained of a true syncytium. The larger vacuoles, which may be intercellular spaces, contain material which gives a positive saliva-resistant P.A.S. reaction and stains with alcian blue and mucicarmine. Glycogen is present in the form of fine intracellular granules. Only small cell remnants are left in the centre of the constrictions, The sheath is most marked and thick in the cartilaginous centra and thins out towards the dilatations almost to vanishing point where these are widest. It is no longer eosinophil, but otherwise gives staining reactions similar to those of the earlier stage. There is again a differentiation of the thick part of the sheath into a narrow, more deeply staining periphery and a wide, paler, homogeneous inner zone.

Electron micrographs of the notochord in the intervertebral regions reveal that most of the large vacuoles are, indeed, intercellular. Secondly, the notochord cells are separated by clearly defined cell membranes (Pl. 3, fig. 9). Some of the large intercellular vacuoles contain microfibrils similar to those present in the sheath. Smaller vacuoles are present inside the cytoplasm and are commonly related to strands of the endoplasmic reticulum. Mitochondria are very scanty. The sheath, about 2μ thick, shows scattered, irregularly arranged microfibrils on an otherwise empty background. There is no external limiting membrane to this sheath which, therefore, is continuous with the matrix surrounding the perichordal cells (Pl. 3, fig. 9).

The notochord sheath in the intravertebral regions is considerably thicker (about 10μ) and encloses scattered cellular debris (Pl. 3, fig. 10). Thus there is no longer a sharp demarcation, internally, of the sheath. The cellular remnants differ in size and the larger ones contain tiny vesicles (Pl. 3, fig. 11). As in the 15-day

stage, the sheath shows two zones, an inner one with few microfibrils and an outer one with a much greater condensation of microfibrils (Pl. 3, fig. 10). Again there is no external limiting membrane to the sheath.

DISCUSSION

These observations on a limited series of rabbit embryos illustrate the comparatively rapid transformation of the notochord from the primitive arrangement as a rod of cells to a stage where continuity of the cells is lost in the regions of the developing centra. In the 13-day stage, the notochord shows the primitive arrangement but by 15 days, a slight intervertebral fusiform expansion associated with intravertebral narrowing is present. By 17 days, continuity of notochord cells is lost in the developing vertebrae, concomitant with a considerable expansion in the intervertebral regions. At least three possible explanations can account for this process of segmentation. Köllicker (1879) considered the mechanism to be one of passive displacement of cells due to pressure exerted upon the notochord during the chondrification of the vertebral bodies. This view received the support of Schaffer (1910) and Dawes (1930). Our study shows that, by the 15-day stage, there is already an increase in the cell population in the intervertebral regions but no constriction of the sheath in the intravertebral regions to suggest that pressure is being exerted from outside at these sites. One alternative mechanism is localized proliferation of the intervertebral notochord cells. Mitoses have been recorded up to the 3·5 mm. stage in human embryos by Prader (1945). Although the present investigation concerns embryos far older than these, it covers the vital period when segmentation first appears, but no mitoses were seen in the notochord cells. A third possibility is migration of cells from the intravertebral to the intervertebral regions. Our material suggests that this may be the first important step in the early segmentation of the notochord but, in the progression of the segmentation, two other factors contribute, degeneration of the remaining cells in the centra and vacuolization in the intervertebral regions.

There is also segmentation of the sheath. The width of the sheath is uniform throughout the notochord in the 13-day stage but, by the 15-day stage, it shows considerable thickening in the intravertebral regions without any increase in width in the intervertebral regions. This difference between the two regions is more marked by the 17-day stage and the sheath is approximately five times thicker in the developing centra than in the regions of the intervertebral discs, where it thins out almost to vanishing point over the notochord dilatations.

The arrangement of the notochord cells is epithelial in character to begin with, which is clearly brought out by the electron micrographs. Cell boundaries are distinct and remain so, even when the appearance changes to a network pattern. There is therefore no true syncytium such as was described—justifiably, on the basis of light microscopy—by Williams (1908) and Dawes (1930), and referred to as 'chorda reticulum' by Peacock (1951). The transformation of the original epithelium into a network arrangement with mucoid material in the intercellular spaces is not unlike the formation of the enamel pulp.

The staining reactions of the sheath indicate that collagenous material is present in

a matrix containing acid mucopolysaccharides. The weak but definite staining with Weigert's elastin in the outer part of the sheath suggests that it contains, in addition, some elastic material. Such a composition is compatible with the finding of microfibrils on electron microscopy, although no definite periodicity of these fibrils has been demonstrated. Electron microscopy showed, furthermore, two definite zones to the sheath in the intravertebral regions in the 15- and 17-day stages, an outer, dense fibrillar and an inner 'emptier' zone corresponding to the deeply staining and the pale, more homogeneous portions of the sheath seen with the light microscope.

With regard to the origin of the sheath, no definite conclusions can be drawn from the present study. Continuity seen, on electron microscopy, between some microfibrils of the sheath and the basal cell membranes of the notochord cells in the 13-day stage would suggest a notochordal rather than a perichordal origin, a view which links up with Duncan's (1957) observation. Also the differentiation of the sheath into the two zones described supports this theory. The absence, on electron microscopy, of any definite external limiting membrane to the sheath does not necessarily negate this view. However, since the sheath is well marked even in the earliest stages examined, younger embryos will have to be investigated for the elucidation of this particular problem.

SUMMARY

1. Descriptions are given of the histology and fine stucture of the notochord in rabbit embryos of 13, 15 and 17 days. Points particularly noted are the general characteristics of the notochord cells, the development of segmentation and the structure and histochemical reactions of the sheath.

2. The notochord cells show an epithelial arrangement in the early stages but later are transformed into a network pattern. Cell boundaries on electron microscopy are distinct in all three stages and there is no evidence of a true syncytium.

3. Only cell debris remains in the intravertebral regions at 17 days.

4. Histochemical reactions of the notochord sheath are described. It is composed of collagenous material in a matrix containing acid mucopolysaccharides.

5. Electron microscopy of the sheath shows the presence of microfibrils which, in the 15- and 17-day stages, are concentrated towards the periphery of the sheath.

6. There is no external limiting membrane to the sheath.

7. The mechanism of segmentation of the notochord and of the origin of the sheath are discussed.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. T.S., 13-day rabbit notochord. The notochord cells show an epithelial arrangement and the sheath is wavy in outline. Outside the sheath are mesenchymal cells forming the perichordal tube. Masson's trichrome, ×770.

Fig. 2. L.S., 15-day rabbit notochord. The section passes through an intervertebral region (I.V.) and a developing centrum (*C.*). In the intervertebral region there is a slight dilatation of the notochord, the cells filling the sheath. In the developing centrum, the notochord cells are fewer and there is a pale interval between them and the most peripheral part of the sheath (arrowed). Azan, ×280.

Fig. 3. L.S., 17-day rabbit notochord. The section passes through an intervertebral region (I.V.) and a developing centrum (*C.*). There is a high degree of vacuolization in or/and between the notochord cells, giving an appearance somewhat like a 'reticulated epithelium'. Only small cell remnants remain in the developing centrum. Azan, ×280.

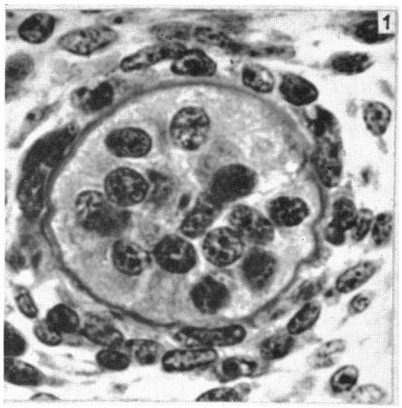
Fig. 4. L.S., 13-day rabbit notochord. Electron micrograph. The nuclei are oval in a transverse direction and the cells are arranged in an epithelial-like pattern. Small vacuoles are present in the cytoplasm. Cell boundaries are clearly seen. The sheath (*S.*) shows no evidence of an external limiting membrane. Palade-fixed, ×2100.

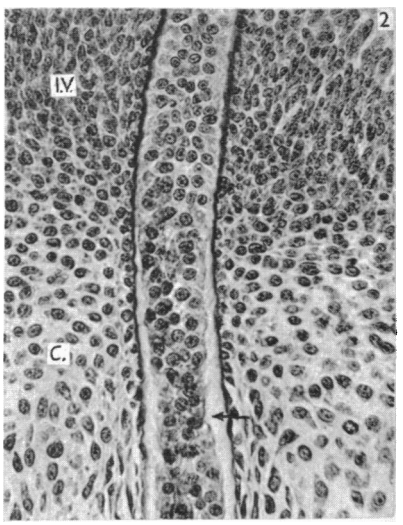
Fig. 5. Thirteen-day rabbit notochord. Electron micrograph. Peripheral parts of the notochord cells, cut obliquely, to show nuclei (*N.*), mitochondria (*M.*) and strands of endoplasmic reticulum (*E.R.*). Part of a cell of the perichordal tube is shown (*T.*). Microfibrils forming the sheath (*S.*) can be seen. Palade-fixed, ×19,000.

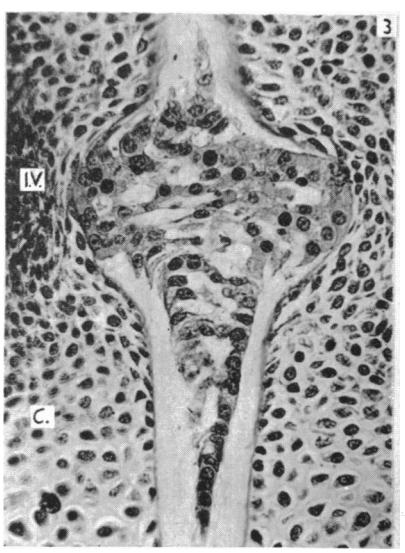
PLATE 2

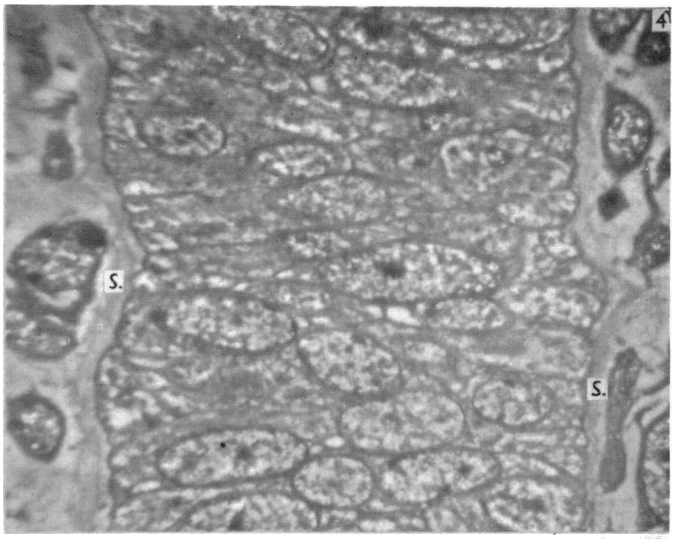
Electron micrographs of 15-day rabbit notochord

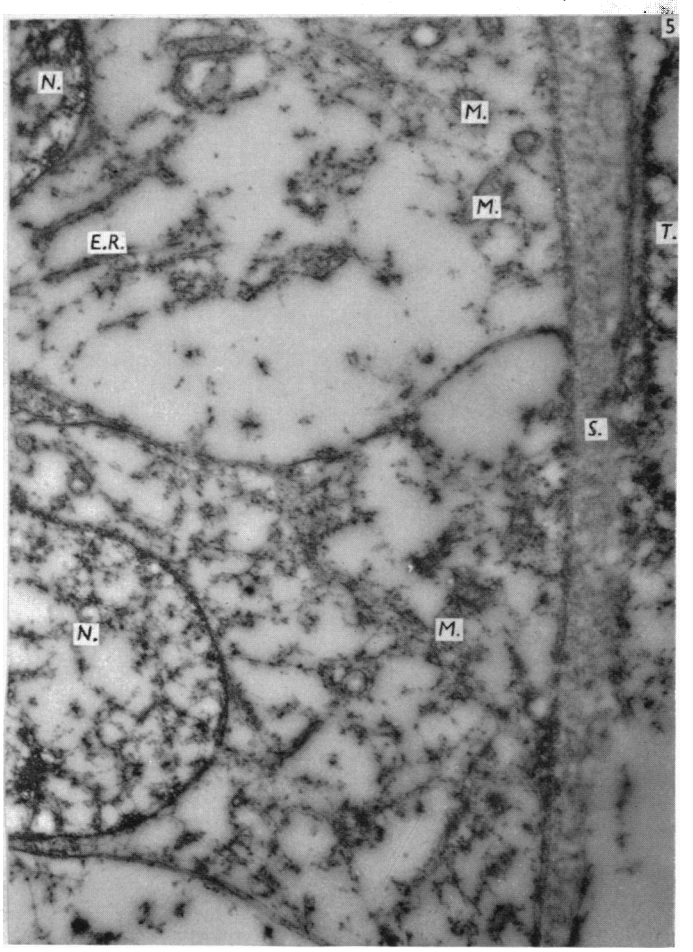
Fig. 6. T.S. of notochord in intravertebral region. The notochord cells show vacuoles (*V.*), and basal spikes (*SP.*) project into the sheath (*S.*) the outer zone of which is composed of densely arranged microfibrils. Outside the sheath are mesenchymal cells of the perichordal tube (*T.*). Palade-fixed, ×2500.











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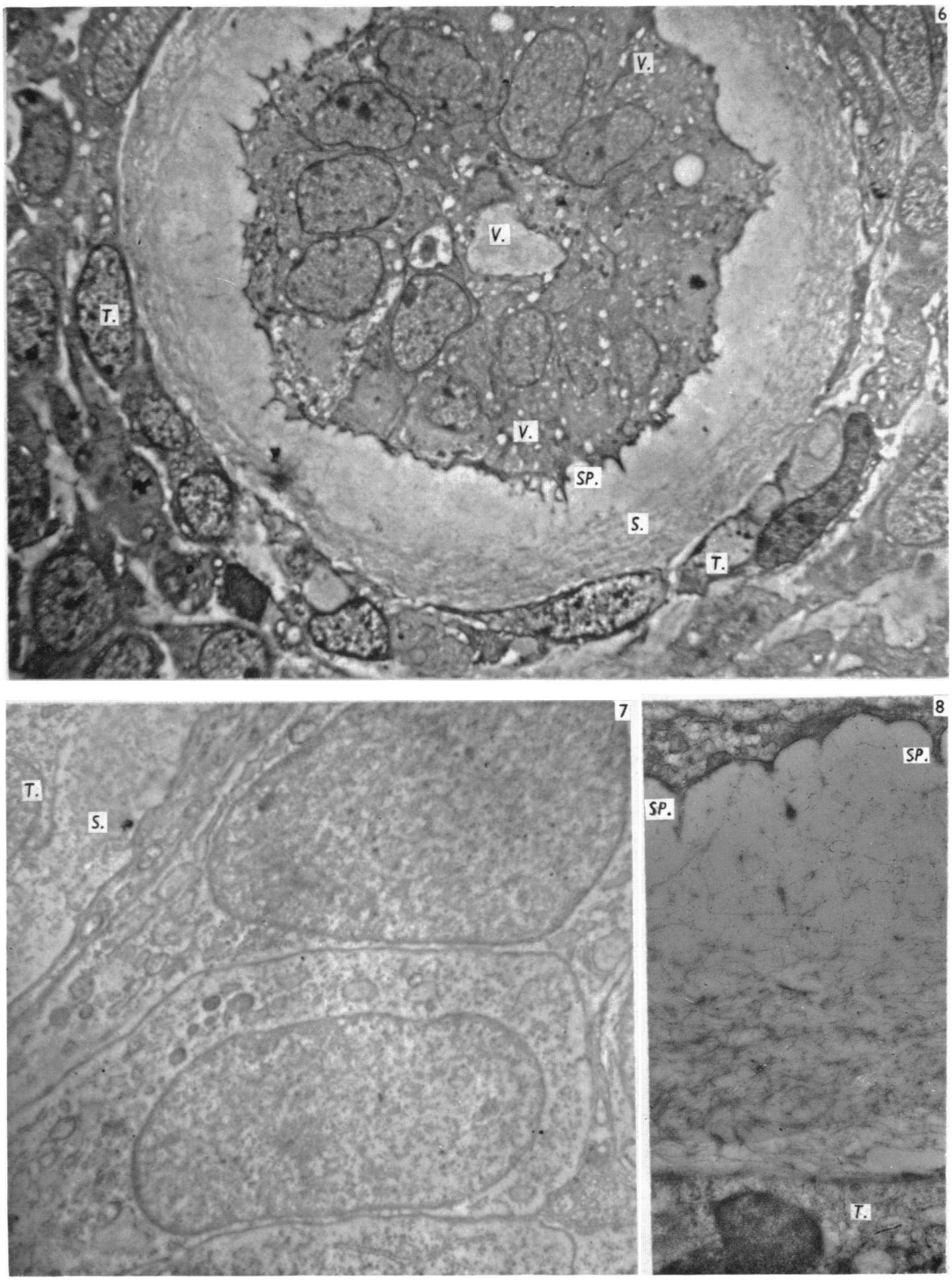

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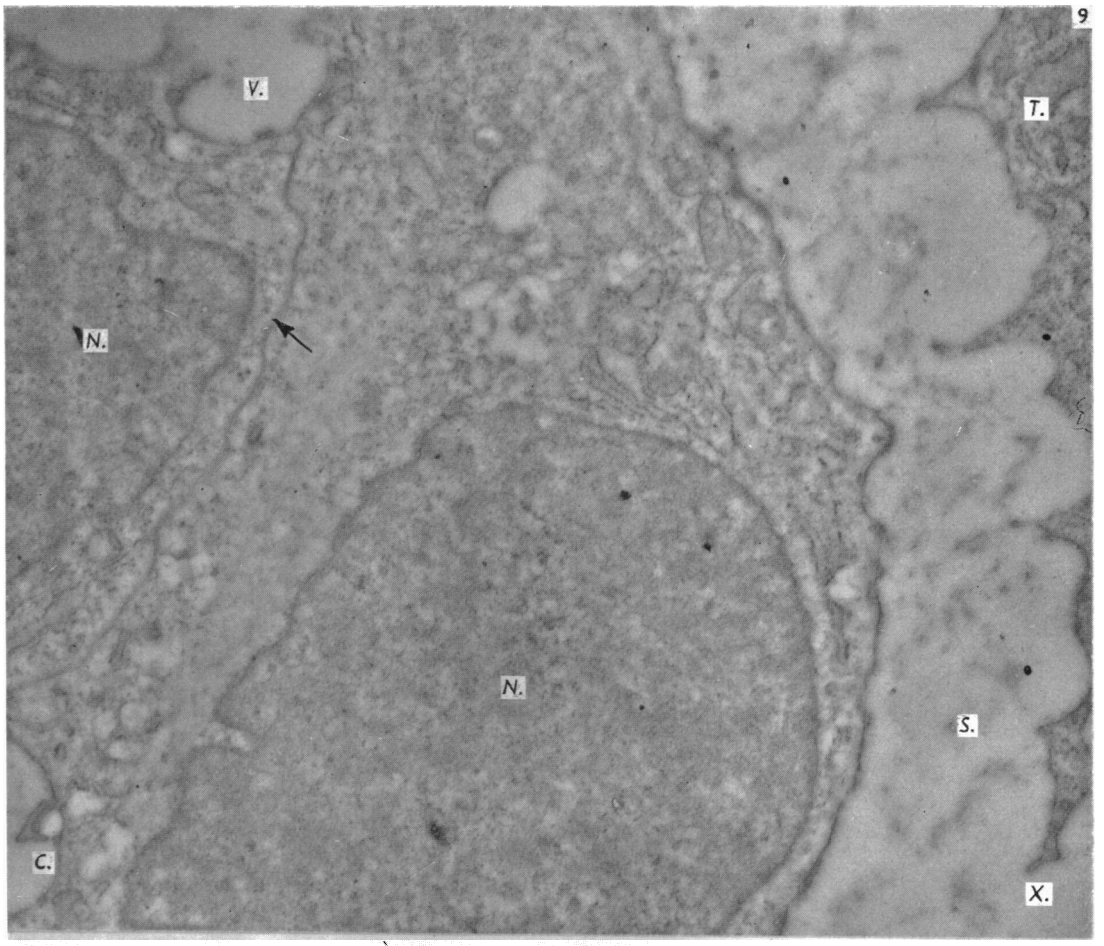

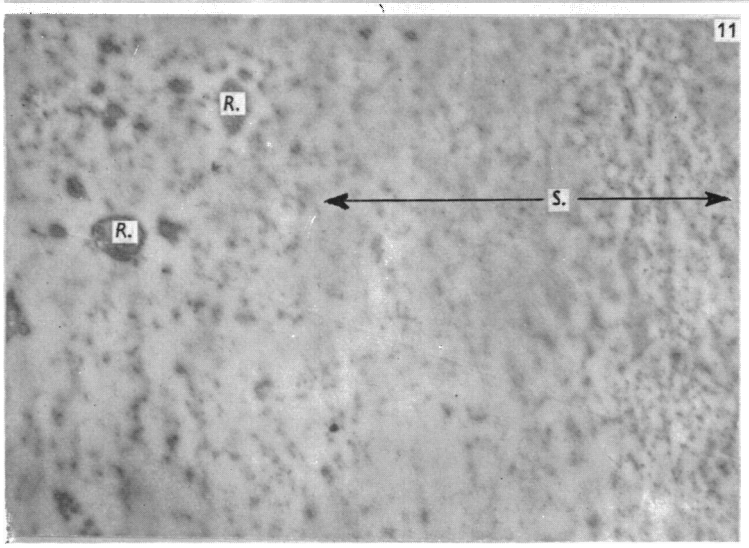

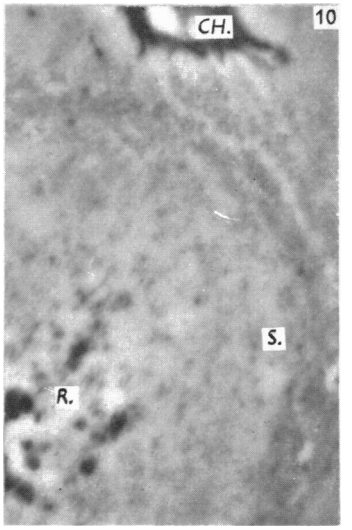

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Fig. 7. Oblique section of notochord in intervertebral region. The sheath (*S.*) shows no clear division into zones. A cell of the perichordal tube (*T.*) is also seen. Dalton-fixed, × 8750.

Fig. 8. L.S. of notochord sheath in intravertebral region to show basal spikes (*SP.*) of the cells, an inner and an outer zone to the sheath, and part of a cell of the perichordal tube (*T.*). Dalton-fixed, × 10,000.

PLATE 3

Electron micrographs of 17-day rabbit notochord

Fig. 9. T.S. of intervertebral region. Parts of two notochord cells and their nuclei (*N.*) are shown, with a distinct cell membrane (arrowed) between the cells. Intercellular vacuoles (*V.*) are shown. Part of a perichordal cell (*T.*) is seen, and below it (*X.*), no evidence of an outer limiting membrane to the sheath (*S.*). Dalton-fixed, × 13,000.

Fig. 10. T.S. of intravertebral region to show the general arrangement of the cell remnants (*R.*) and the sheath (*S.*). Outside the sheath there is a cartilage cell (*CH.*) of the developing centrum. Palade-fixed, × 3000.

Fig. 11. T.S. of intravertebral region of notochord showing remnants of notochord cells (*R.*), and notochord sheath (*S.*) with well-marked inner and outer zone. Palade-fixed, × 8500.