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I hereby recommend that the thesis prepared under my supervision by Lawrence Bennett Hobson

entitled ON THE ULTRASTRUCTURE OF THE NEURAL PLATE AND TUBE OF THE EARLY CHICK EMBRYO, WITH NOTES ON THE EFFECTS OF DEHYDRATION

be accepted as fulfilling this part of the requirements for the degree of Doctor of Philosophy

Approved by:

D. L. Wiesman

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ON THE ULTRASTRUCTURE OF THE NEURAL PLATE AND TUBE OF THE EARLY CHICK EMBRYO, WITH NOTES ON THE EFFECTS OF DEHYDRATION

A dissertation submitted to the

Graduate School
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by

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TABLE OF CONTENTS

INTRODUCTION

The investigation of the submicroscopic structure of living tissues by means of polarization microscopy has revealed many details of protoplasmic organization. Within the last few years attention has been given especially to neural structures by F. O. Schmitt and his co-workers (Bear, Schmitt and Young, '37a,b; Schmitt and Bear, '39; Schmitt, '36; Schmitt and Chinn, '37; and Chinn, '38) and by W. J. Schmidt ('37a). These studies have dealt, however, with adult vertebrate and invertebrate material, and principally with the nerve fiber. The properties of the adult neuron cell body have been described by Chinn ('37).

Very little use has been made of polarization microscopy in the study of embryological material. The studies of Runnström ('31) on echinoderm embryos and of Schmidt ('24) on Chirodota larvae were concerned primarily with the development of skeletal elements and only indirectly with the associated living tissue. Vlès ('34) has studied fertilized and cleaving sea-urchin eggs and has correlated cleavage with variations in the refractive index. He believes that the protoplasm exhibits birefringence of the molecular type which is probably due to a protein structure alone or associated with organized lipoid. No studies, however, have been made upon the nervous systems of early vertebrate embryos.

The present investigations were undertaken in the hope that they might afford some explanation, in terms of molecular

and colloidal mechanics, of changes in the early neural development and thus narrow the gap between visible effects and invisible causes of embryological processes. The early stages in the development of the nervous system are suitable for study by polarization optics, and the microscopical details of the process have been thoroughly investigated.

An extensive search for satisfactory material for this study revealed disadvantages in the use of embryos of many common laboratory animals. It was thought best to use a form which can be examined in the living or surviving condition and one whose development is already well known. Moreover, the presence of pigment in an embryo prevents its study by transmitted light, and yolk granules are so highly birefringent that they mask other optical activity. This makes it impossible to use embryos of the Amphibia, which contain large amounts of yolk within their cells.

The developing nervous system of the chick, however, meets the above requirements, and the cytological constituents of the early neural tube have been studied extensively by Cowdry ('14). The studies reported here deal with the early stages in the development of the chick from the time of laying until the end of the second day of incubation, before myelinization has begun, and were made principally at magnifications of 400 diameters or less. Some study of cellular detail was carried out with oil immersion objectives,

but the cytological details require further amplification.

During the investigation of the birefringence of the nervous system, changes in the position of the neural folds were observed on immersing the embryos in dehydrating agents. This suggested a short study of the role of hydration in the closure of the neural folds. These movements in the formation of the tube have been studied by Gurwitsch ('14) who believed them to be due to cellular movements, and by Glaser ('14, '16) and Giersberg ('24) by whom a role was assigned to hydration. The question is still unsettled, however, and the correlation of movements and structure is lacking.

MATERIALS AND METHODS[1]

Eggs were obtained from a small flock of Rhode Island Red chickens and incubated in a gas incubator, the temperature of which was kept constant within three degrees of 103° Fahrenheit. The eggs were kept at room temperature until incubation, which was always begun within four days of receiving them.

Six embryos of various ages were examined at 38° Centigrade and again at 23° Centigrade to determine the effects of cooling on their birefringent properties. There were no

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[1]The publications of Schmidt ('24,'35,'37a) and of A. Köhler ('28) contain descriptions of the methods of polarization microscopy as applied to biological material.

detectible changes and subsequent examinations were made at room temperature. This made it unnecessary to use a slide warmer which had caused uneven heating of the slide and produced birefringence in the glass.

Likewise, the use of balanced salt solutions and of fluid from the subgerminal space of older embryos was discontinued when it became evident that they had no advantages over a 0.75% NaCl solution. When kept at room temperature in the simple saline solution the embryos have a regular, though slowed, heart rate for several hours. The intense light used for the microscopic examination is detrimental but embryos survive for a period of time longer than that required for the examination.

Embryos were occasionally fixed before or after removal from the egg, and various reagents were tried, the most satisfactory being a solution of 10% formaldehyde in 0.5% acetic acid. With this fixative distortion is minimal since the relative concentration of formaldehyde and acid is such as to prevent shrinking or swelling, and the material is not discolored as it is with mixtures containing picric acid. The fixation has no immediate effect upon the birefringent properties (Chinn,'38) but the opacity of the fixed tissue makes examination by polarized light more difficult.

To remove lipoid constituents, fixed or fresh material was extracted with several changes of alcohol and acetone or either alone. Embryos and isolated neural tubes were

soaked in each solvent for thirty minutes or more although extraction was complete in a shorter period. Removal was equally effective from material fixed in formaldehyde-acetic acid and in mercuric chloride.

Treatment with imbibition fluids of various refractive indexes was carried on both before and after fixation. After removal of the lipoids many fluids are available, but where the embryos are examined whole a limit is imposed by the increasing opacity of the tissue as the refractive index varies from that giving minimum form birefringence. Alcohol-xylene, xylene-methylene iodide mixtures, and glycerine in varying concentration were used to obtain refractive indexes from 1.332 to 1.74.

With embryos from which lipoids had not been extracted, glycerine, sucrose, and glucose were used in varying concentration. This limits the maximum refractive index for such imbibitions to 1.47 and often causes marked shrinkage. Different concentrations of sodium chloride and calcium chloride were also tried.

Embryos were examined in toto from all possible angles, but examinations were usually made in the dorso-ventral plane. For this purpose the embryos were examined in small watch glasses or depression slides which allow the use of relatively large volumes of fluid and permit easy manipulation of the material. Besides whole embryos, teased preparations and sections in the three principal planes were examined for

cytological detail.

When it was desired to examine the neural structures free from the adjacent tissues, dissections of the embryonic area were made after removal from the egg. The work was done under a dissecting binocular microscope and the area pellucida was anchored to the bottom of a fluid-filled watch glass or depression slide by means of a glass ring laid around its periphery. Watchmaker's forceps and fine glass needles were used for the dissecting. Dissections of surviving embryos were rapid and crude; those of fixed embryos were complete even to the removal of the notochord in many cases.

Examinations were made with a Leitz petrological microscope, using a low voltage tungsten ribbon lamp at maximum intensity. For the study of the slight amounts of birefringence present in this material the usual interference plates or sensitizers were employed, and use was also made of objectives showing varying amounts of strain birefringence. As Schmidt ('37b) and Monné ('39) have shown, these work well as qualitative substitutes for the Köhler compensator.

Since a Köhler compensator was not available for measuring retardations, a 1/16th wave length mica plate mounted for rotation upon the stage was used instead. As the object was supported above the stage the attendant separation from the condenser makes the use of high power objectives difficult. The measurements are accurate, however, within the

limits of the mica plate, and each determination is the average of five readings. Measurements of dimensions were made with an ocular micrometer.

The refractive indexes of the imbibition fluids were routinely determined by means of a stage refractometer (see Chamot and Mason, '31). Frequent checks were made with an Abbé refractometer and the latter was used for all of the more important determinations.

RESULTS

Neural Tube

The study was begun with examination of the neural tubes of 103 embryos beyond the stage of neural tube closure. This served as a basis for examination of earlier and later stages, but the development of the brain region and its vesicles will not be discussed in this paper.

When an embryo of about ten somites is examined with crossed prisms and an objective set to act as a sensitizer (one plane of vibration at 45° to those of the prisms) its optical characteristics can be well seen. As the stage is rotated through 360° the neural tube (spinal cord) will become light in two positions, those at which its long axis forms an angle of 90° with the plane of vibration of the slow component of the objective. In these positions the tube will appear brightest toward its rostral end, and the

medial half will be more intensely lighted. Rotation of the stage through 180° brings the tube to the extinction position and it appears darker than its surroundings, the darkest portions being rostral and medial.

In all such surviving embryos the parts of the closed tube show some slight birefringence. The optic axis of the tube as a whole lies radial to it, and birefringence is maximal in the walls of the tube when examined from above, the roof and floor when examined from the sides.

Without exception the slower component vibrates in a radial direction and thus along the long axis of the cells making up the walls of the tube. In regions where the tube is not distorted by vesicle and sulcus formation the walls are, therefore, negative with respect to the long axis of the tube. Birefringence is usually so slight that this must be determined by using 1/10th or 1/16th wave length rotating plates, or by observations with objectives used as compensators. In a few older embryos a Rot I plate can be used.

The amount of birefringence varies with the thickness of the tube but there is also visibly greater birefringence (in some instances twice as great) in the medial half than in the outer half. This is not due to thickness variations alone, as indicated by a greater difference between inner and outer halves than between thick anterior and thin posterior regions of the tube.

Examination of the cellular components in the living

state by the methods available for this study is difficult. It shows, however, that birefringence is present in nuclear and plasma membranes to much greater extent than in the cytoplasm itself. The membranes are positive with respect to the tangent of their surfaces, and the cytoplasm is often slightly positive with respect to the long axis of the cell. It is optically empty when first examined except as it contains birefringent yolk globules, but becomes granular slowly. Cells in mitosis are more birefringent in the region of the spindle and this may account for the greater birefringence in the medial half of the tube where they are more abundant.

In many instances considerable birefringence is present in the internal and external limiting membranes. In these the slower of the two components vibrates parallel to the surfaces, i.e., the character is the opposite of the neural tube as a whole, and the optic axis lies perpendicular to them.

Fixation increases the difficulty of examination of this thick material by making it opaque, but it does retain the anisotropy of neural structures for a month or more. Embryos kept in the formaldehyde-acetic acid solution for a year became discolored (gray to dark brown) and very opaque but the character of the birefringence was unchanged.[2]

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[2]Treatment with glycerine, however, produced no reversal of sign, and it thus seems that there is a slow disintegration of the lipoid components.

Imbibition of fresh or fixed neural tubes with fat solvents alters the strength of birefringence but does not reverse its sign, i.e., the tubes remain negative with respect to their long axes. The same is true of the cellular components as seen in teased or sectioned material. Nuclear and plasma membranes become especially active and can be distinguished in whole tubes at relatively low magnifications.

Treatment of the neural tube with glycerine, sucrose or glucose solutions causes changes both in the strength and sign of its birefringence; it becomes positive with respect to length as its character reverses. The shift is chiefly in the plasma and nuclear membranes but involves the cytoplasm also. This reversal of sign may be termed metatropic if the change is thought of as one from the positive character of the cells composing the walls of the tube to a negative one.[3] The limiting membranes retain their original character but the strength of their birefringence varies in different media.

Electrolyte solutions also cause the metatropic reaction and alter the strength of birefringence as their concentration is increased. Sodium chloride, potassium chloride, calcium chloride, and sodium bicarbonate are all effective but they produce an increased opacity and in low concentrations

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[3] This preserves the original meaning of the term as introduced by Göthlin ('13) and as used by Bear and Schmitt ('37).

lead to rapid disintegration.

The metatropic reaction of the walls (also the roof and floor) of the neural tube indicates the presence of both form and intrinsic birefringence there. The constituents of the tube wall are so arranged that the slow component of the form birefringence vibrates perpendicular to the long axis of the tube, and in the living state its strength is great enough to mask the presence of the intrinsic birefringence. The slow component of the latter is in a plane paralleling the long axis of the tube. Figure 2 shows these optical properties.

In the course of examinations of neural tubes surviving in 0.75% NaCl solutions it was noticed that the strength of birefringence increased with time. It is greatest when the cells become highly granular and disintegration is imminent. When tissues are killed by heat, exposure to strong sunlight, or prolonged standing at room temperature, there is a similar increase in the strength of birefringence shortly after the heart beat stops. Treatment of such slowly killed tissues with fat solvents gives results identical with those on fresh neural tubes, but when glycerine imbibition is attempted a greater concentration is required to cause the metatropic reaction and the reversed birefringence remains weak; in some cases the reversal does not occur at all.

This loss of the metatropic reaction follows the des-

truction of one or the other forms of birefringence. Here the intrinsic birefringence disappears during the changes accompanying death of the cell and leaves only the form birefringence. This disappearance does not occur, however, when fixation by suitable agents is prompt.

Quantitative studies of the birefringence of the neural tube were made in an attempt to detect regular variations during the course of early development. With the methods of measurement available there were no significant changes in a series of 35 embryos. It is possible, however, to determine the approximate strength of birefringence of the neural tubes. Measurements on embryos immediately after incubation give a birefringence ($n_a - n_o$) of the order of 4×10^{-5}. The measurements made on forty surviving embryos showed a range from 9.0×10^{-6} to 1.2×10^{-4}, but the variations are not proportional to the age. The extremes were embryos in which the accuracy of the measurement of the thickness of tissue observed is questionable.

The retardation (Γ) increases as the size of the neural tube increases with age and as measurements are made more rostrally. The corresponding increase in the thickness of material (d), however, is such that the birefringence remains at the same value. Table 1 (page 13) gives the measurements made on the neural tube at the levels of somites 2 and 6 of three embryos of different ages. The variations in the values for birefringence are not significant and the

apparent decrease in the level of somite 6 is not substantiated by observations on other embryos. This is of especial interest since Cowdry ('14) has shown that fibrils (neurofibrils) develop in the cytoplasm of the neural tube cells at the levels of somites 2 and 6 before the 23 somite stage. Their appearance is not accompanied by a marked increase in the birefringence of these cells.

TABLE 1

Measurements of Birefringence of Unfixed Neural Tubes

	Somite level 2			Somite level 6		
Ages in somites	Γ	d.	$n_a - n_o$	Γ	d.	$n_a - n_o$
8	8.7 mμ	180μ	4.8×10^{-5}	6.9 mμ	120μ	5.7×10^{-5}
13	5.4 mμ	140μ	3.9×10^{-5}	5.1 mμ	120μ	4.3×10^{-5}
23	14.1 mμ	290μ	4.9×10^{-5}	7.4 mμ	195μ	3.8×10^{-5}

Imbibition curves show no significant variations for the ages studied but they reveal certain facts about the protein and lipoid constituents of the tissue (Fig. 1).

In determining the point of reversal of the character of birefringence (Fig. 1, curve A), values for the refractive index were lower the more rapidly readings were made. If the neural tube remains for a total of thirty minutes in 5% glycerine, birefringence becomes zero in 25% glycerine (n = 1.364). With reduction of the time to five minutes and gentle agitation

of the solution, the change occurs in 6% glycerine (n = 1.34). (Table 2.) A neural tube imbibed with 10% glycerine (n= 1.35)

TABLE 2

Reversal Point of Unfixed Neural Tubes in Glycerine

Time in 10% glycerine	Point of reversal
1 hour	25% : n = 1.37
30 minutes	25-% : n = 1.36
8 minutes	10-% : n = 1.347
5 minutes	Already reversed (Reverses at 5% : n = 1.339)

for ten minutes shows a reversal of sign and becomes positive with respect to length. On standing in the solution the tube returns to its original character, i.e., negative with respect to length, and requires a higher concentration to produce the metatropic shift.

In Figure 1 a series of retardation values of neural tubes is plotted against the refractive indexes of the fluids in which they were measured (Ambronn-Frey imbibition technique). The tissues upon which the determinations for curve B and for the lower end of curve A were made were fixed in formaldehyde-acetic acid mixture, and in many cases the neural tubes were dissected free from the adjacent tissues. Lipoids were extracted from the tubes for curve B by soaking them for thirty minutes in alcohol and in acetone, and fixed tissues were imbibed for thirty minutes in the different

fluids. Measurements were made at the level of the second somite through the walls of the tube, the thickness of which was then measured.

The exact values for the curves are not significant, but the general characteristics have been substantiated by other series in which the thickness of the tissue was not measured so that only Γ could be calculated. The curves agree generally with those obtained by Chinn ('38) for the sheath of the frog ganglion cells, and the values of minimal form birefringence are within the range of those obtained by Bear, Schmitt and Young ('37b) for the axis cylinder of the squid. It will be noted, however, that there is no neuron sheath in the embryonic tissue studied here and that there is a metatropic reaction not present in the axis cylinder.

Table 3 (page 16) presents the data of another and larger series of embryos upon which less extensive observations were made. It differs also from the graph in that the unextracted embryos were not fixed before being placed in glycerine. The averages for the measurements at the second somite level of this series are superimposed upon the graph, and the data in both table and graph have been separated into a younger and a slightly older series. There are no significant differences in these two groups, nor in the others studied.

Measurements on embryos in sucrose and glucose solutions agree with those in glycerine. Calcium chloride and

sodium chloride solutions cause isotropy at n = 1.335 and at about n = 1.35 respectively. An attempt to extend the lower end of curve A by means of Carbowax imbibition was unsuccessful.

TABLE 3

Changes in $n_a - n_o$ of Neural Tube During Imbibitions

	Somite level 2	Somite level 6
In saline (n = 1.34)		
Early (0 to 15 somites)	4.2* (5)**	4.0 (5)
Late (16 to 30 somites)	4.1 (5)	1.4 (1)?
Average	4.2 (10)	3.6 (6)?
Unextracted into glycerine*** (n = 1.47)		
Early (0 to 15 somites)	8.2 (4)	8.1 (1)
Late (16 to 30 somites)	9.4 (1)	7.8 (1)
Average	8.5 (5)	7.9 (2)
Lipoid-extracted in xylene (n = 1.5)		
Early (0 to 15 somites)	5.1 (3)	5.2 (3)
Late (16 to 30 somites)	2.8 (2)	3.5 (1)
Average	4.1 (5)	4.8 (4)

* All values x 10^{-5}.
** Number of embryos measured given in parentheses.
*** Character reversed.

The quantitative analysis of the neural tube shows that the form and intrinsic birefringence are due respectively to protein and to lipoid components. The minimum point of curve B (Fig. 1) is at about n = 1.57 and is the mean refractive index of the particles responsible for the form birefringence (micelles). This is approximately the refractive index of 1.576 found for the protein micelles of myosin (Weber,'35), of 1.58 for nerve sheath proteins (Chinn and Schmitt,'37), and of 1.57 to 1.60 for the axis cylinder proteins (Bear, Schmitt and Young,'37b). The form birefringence is thus due to oriented protein micelles, as it is in other living tissue.

The lipoid source of intrinsic birefringence is indicated by its disappearance on extraction with fat solvents. The role of lipoids in the ultrastructure of nerve fibers and cell bodies has been demonstrated by Schmitt and Bear ('39) and Chinn ('38), and it seems probable that here, as well, lipoids oriented so that the slow component of their birefringence is perpendicular to that of the protein, are important. This is also indicated by the loss of intrinsic birefringence at the time of cell death, since Lepeschkin ('37) has produced evidence that lipoid disorientation is an early result of cell death. The low concentration of glycerine (6%) necessary to produce the metatropic reaction in the neural tube, as compared to the sheath of differentiated nerve cells (30% and 50%) (Chinn,'38), indicates a higher concentration of oriented lipoids in the former. This has not been proved by

chemical analysis and is probably apparent rather than real.

Neural Plate

Birefringence is present in the neural tissues before the formation of the neural tube, although less intense than later. A study of these earlier steps in the formation of the neural tube was undertaken in 27 embryos.

The mesoderm and endoderm close below the neural plate make it difficult to examine the ectoderm alone, and dissections of the plate from the subjacent structures are necessary. Observations were also made with the embryo oriented so that the plate could be seen from the side.

The amount of birefringence in the surviving neural plate is too small to measure. Viewed from the surface it is usually isotropic but appears very weakly positive with respect to length in a few (three) embryos. Seen from the side it is weakly negative with respect to the long axis of the embryo.

The opacity of fixed specimens makes it impossible to detect birefringence in the neural plate unless the tissue is subsequently cleared. When this is done in the preparation of lipoid-extracted embryos the sign of the birefringence is reversed so that the plate is now negative with respect to the long axis of the embryo seen both from above and from the side.

In 100% glycerine the neural plate is birefringent, and if it lies flat so that observation is directly from above, it is negative with respect to its longer axis in most cases but positive in a few. It is positive with respect to its length when seen from the side. The plate shows the same characteristics from the time of its appearance in the head fold stage until it is no longer seen flat, at about the 10 somite stage, but the retardation is always slight.

Optical activity is thus present even when the neural plate is viewed along what will later become the optic axis of the neural tube, i.e., along a line perpendicular to the surface. This anisotropy is due to form birefringence alone, or to form birefringence and intrinsic birefringence with slow component vibrating in the same plane, since its character is the same in imbibed embryos both before and after lipoid-extraction. The opposite sign of the fresh neural plate may be due to subjacent tissues since these cannot be thoroughly removed by the rapid dissection used on the three embryos in which it was seen.

Neural Folds

As the edges of the neural plate elevate to form the neural folds the character of the region changes so that, as the tissue of the plate is seen from what was originally its side, it appears negative with respect to the long axis of the fold and of the embryo in the living state. The

appearance is complicated by the simultaneous elevation of the adjacent epidermal layer, which is optically inactive when seen in profile alive but becomes active in imbibition fluids. Fixation preserves the optical character of the folds but makes observation more difficult.

Extraction with fat solvents increases the strength of birefringence with no reversal of sign. The epidermal folds become anisotropic and positive with respect to their length, viz., the opposite of the neural folds. Imbibition curves have the same shape as curve B (Fig. 1) and the values of birefringence are not significantly different from them (see the values for 3 to 4 somites stages on this curve).

Glycerine imbibition of unextracted embryos reverses the character of the neural fold so that it becomes positive with respect to length, but the adjacent epidermis is of the opposite sign, i.e., negative with respect to the length of the fold. This fact and the tendency of the embryo to wrinkle during dehydration cause many complicated and confusing appearances as the result of chance variation in the amount of neural and epidermal activity. With the completely closed tube the epidermis is leveled above it so that the phenomenon does not appear. These are seen both in the caudal portions of embryos of about the 10 somite stage and in the more rostral regions of younger ones. Figure 3 is a diagrammatic representation of such a region.

The elevation and closure of the neural folds to form

the tube does not, therefore, change the nature of the birefringence seen in the plate from the side. The mixture of form and intrinsic birefringence of the later tube is already established in the open plate so that the lipoid and protein components are already oriented.

Primitive Streak

Study of the development of the neural tissues has demonstrated their origin from the ectoderm of the primitive streak area (Wetzel,'29; Kopsch,'34; and Rawles,'36). However, the thinness of the area, its tendency to curl and fold, and the firm adherence of subjacent structures which prevents isolation of the ectoderm, make study of the region with polarized light difficult.

Birefringence is very slight in this region observed whole in 0.75% NaCl solution. Down the center of the streak it is often weakly positive with respect to length and somewhat more lateralward it is negative. The part of this due to the ectoderm could not be determined, and fixed specimens are too opaque to show any activity except for the numerous adherent yolk granules.

Fixation, lipoid-extraction, and glycerine imbibition cause wrinkling and confusing alterations in the region. In general, however, fluids of greater refractive index, with or without lipoid extraction, seem to preserve the positive

character of the central streak. More lateralward is an area the character of which is reversed by glycerine imbibition (without extraction) so that it becomes positive. This strip varies in width and may be absent; its presence has not been correlated with age or the exact tissue responsible, although this seems to be below the ectoderm. The remainder of the streak area remains weakly negative with respect to length, or isotropic, except where thrown into folds. Seen from the side in 100% glycerine, the streak appears very weakly positive with respect to length.

Lipoid-extracted embryos retain the characteristics of the fresh ones, i.e., a central positive streak and a negative lateral area. The birefringence, however, is weak.

These results do not indicate the structure of ectoderm in the primitive streak area. If it shows optical activity it is only slightly greater than that of the surrounding epidermis. No constant or characteristic properties were noted in the region of the primitive pit, although folds here are anisotropic in the same manner as in the remainder of the ectoderm.

Unsuccessful attempts were made in surviving embryos to produce birefringence in extra-embryonic, neural plate, and primitive streak areas by stretching and by pressure. The friability of tissues in glycerine and the rigidity of extracted specimens limits the possible manipulations, but in these also external changes are ineffective except as the

tissue is thrown into folds so that epithelial layers are seen in profile. The retardation in these folds of epidermis of the same height as the neural folds is not as great as in the latter. Cellular birefringence as seen under higher powers is also unaltered by mechanical changes.

Non-neural Structures

Detailed consideration of non-neural structures in the embryo lies outside the scope of this study, but a few are important in connection with neural elements. There are non-cellular membranes in the embryo (internal and external limiting membranes of the tube, the membrane surrounding the notochord, and the surface "pellicle" over epithelial structures) which are optically active. These are positive with respect to a tangent to their surface regardless of imbibition but are isotropic when seen from above.

The somites in the stages studied are composed of radially arranged cells which are positive with respect to length in the living and lipoid-extracted embryos, negative in unextracted ones in 100% glycerine. The cellular components are thus optically similar to those of the neural tube.

The notochord itself is very weakly birefringent or isotropic in the living embryo but appears negative with respect to length in balsam and positive in glycerine, if previously unextracted. In both of the latter retardation is slight. Heavy epithelial folds such as the gill pouches and

the gut walls of older embryos have optical activity of the same character as the neural tube but of less strength.

The cells of the somites, notochord, gut wall, etc., show form and intrinsic birefringence similar to those of the neural tube but of less strength. The non-cellular membranes, however, differ in the nature or arrangement of their constituents. Since they fail to show the metatropic shift they either lack intrinsic birefringence or, if present, it has its slower component in the same plane as that of the form birefringence.

The myocardium shows marked optical activity with the slow component tangential to the layer, i.e., negative with respect to radius. This is true in the living, lipoid-extracted, and glycerine-imbibed embryos. Although quantitative studies were not made, the evidence strongly suggests that this birefringence is that of the myosin fibrils, the ultrastructure of which has been described by Weber ('35) and others.

The epidermis with optic axis perpendicular to its surface shows the metatropic shift, but the planes of vibration of slow and rapid components are the reverse of those seen in neural structures. In epidermal folds, either artifacts or those produced by neural tube closure, the slow component of the form (protein) birefringence is tangential to the epithelial surface, that of the intrinsic birefringence is perpendicular to it.

With the exception of the primitive streak area, it is possible by dissection and careful optical separation to discriminate between the activity of adjacent structures. One source of error is the apparent activity seen in an area lighted by reflection from adjacent active material. This accounts in large part for the difficulties encountered in the use of yolk-rich embryos.

Dehydration

In the course of an imbibition series in which unfixed embryos were treated with glycerine it was noted that the part of the spinal cord or brain in which the folds had recently closed to form the neural tube became reopened. This accompanied the shrinkage of the embryo, and the amount of separation of the folds roughly paralleled the extent of dehydration. A short series of experiments was then performed to determine the nature of this change and its relation to the ultrastructure of the neural folds.

Using an embryo of 6 somites with neural tube complete from a small anterior neuropore to somite 5, glycerine was added dropwise to the saline solution, which was gently agitated. A gradual opening of the folds resulted, and at 5% glycerine the folds had opened as far anteriorly as somite 1; at 50% the entire tube was open. The tube of an embryo of 12 somites similarly opened from a level 400 μ caudad to the last somite up to somite 11 on imbibition with 100% glyc-

erine. Although distortion was great the tube remained closed above this level.

Similar results were obtained on ten embryos of 3 to 20 somites. Careful observation before treatment shows that the folds open only so far as the fusion of opposite sides is incomplete. This is shown by the persistence of a visible mid-dorsal seam. In all cases the shrinkage of the embryo with glycerine is less evident in the neural structures than in others, but measurements show a definite shortening of the tube (about 20%) when bending is not too marked.

To show that this failure to open after fusion is not the result of cellular cohesion between the folds, a fine glass needle was inserted into the neurocoele of three embryos in various stages of development (9, 15 and 33 somites) and the roof of the tube ripped open. The walls separate a little after the operation but treatment with glycerine causes only slightly greater spreading than in unoperated controls. In the oldest embryo the folds were obviously more closely approximated following the application of glycerine than immediately before.

Since the principle action of glycerine upon the embryo is dehydration, these effects probably result from the removal of water from some part of it. At the time of complete closure of the neural folds the epidermis loses its connections with them, and the failure of the folds to separate after tube closure might result from the loss of this attach-

ment. If shrinkage of the thin epidermis could exert enough lateral force it might open the folds to which it is attached. However, embryos in which the neural tube has been separated from the surrounding epidermis and mesenchyme by a cut parallel to it give the same results.

Another possibility is that of differential dehydration of the neural tube and folds by glycerine acting upon their outer surfaces, i.e., through the external limiting membrane, more rapidly than through the internal membrane. When a tiny droplet of glycerine is added to the neural groove of an embryo before it is removed from the egg, and with the vitelline membrane intact, the folds open rather than close more rapidly. The latter would be expected if the dehydration were a differential one in the sense of a local removal of water from the tube wall in which it was previously uniformly distributed. The results support the existence of a gradient of imbibition already established as closure occurs and based upon an orderly arrangement of submicroscopic particles which the birefringent properties of the tube wall prove exists.

Immersion of embryos in a 20% NaCl solution causes rostro-caudal shrinkage of about 15% and the neural folds separate as with glycerine (as much as ten times the original distance apart). Concentrated formaldehyde, however, causes a rostro-caudal shrinkage of about the same amount without any attendant fold separation.

Fixation in formaldehyde-acetic acid solution abolishes the reaction, and exposure to strong light for one hour (sufficient to kill the embryo) reduces the separating effects to a minimum. The mechanism in the two cases is different, however; death destroys the lipoid component or its organization and fixation preserves it as shown by polarization analysis. Fixation may further alter the ultrastructure of the tube by changing the interrelations of the micelles.

Careful observation of the folds during opening shows that they reverse the movements made in closing. There is a simultaneous lateralward motion with the notochord as "hinge" and a flattening or "uncurling" so that the end result is a flattened plate. The less completely separated folds show a condition which corresponds to some stage in closure.

Attempts to hasten fold closure by immersion of the embryo in NaCl solutions of less than 0.75% and in distilled water during examination were unsuccessful; the folds showed no movements and disintegration was rapid. Further studies were unfortunately prevented by failure of the egg supply for the season.

DISCUSSION

The significance of birefringence in the embryonic neural system studied here depends largely upon its source. It

might be due to properties common to all epithelial cells like those of which it is composed, or to special properties characteristic of neural structures. Both factors are probably present.

Schmidt ('24a, '37a,b) and Frey-Wyssling ('38) review in detail the submicroscopic structure and the optical properties of different tissue and cellular components. Birefringence has been described in almost all parts of the cell: nuclear and plasma membranes, the chromosomes, spindle fibers, filamentous mitochondria, and "structureless" protoplasm, as well as more specialized cilia, vacuolar membranes, intracellular fibrils, and non-living inclusions. The embryonic nerve cells contain inclusions of yolk granules or globules, but they are distinct and not numerous enough to confuse the birefringence of the living material.

The plasma membrane of the embryonic nerve cells has the same optical properties and probably the protein-lipoid structure described for other plasma membranes, e.g., that of the erythrocyte (Schmitt, Bear and Ponder, '37) and of the adult neuron perikaryon (Chinn,'38). The nuclear membrane also shows the metatropic reaction which is not always present in that of other cells (see Monné,'39). These two membranes are not characteristic of the developing neural tissue but they account for a considerable part of the optical activity of the folds and tube.

The cytoplasm and its contents are also birefringent

to a slight degree. The activity described here in the region of the mitotic spindle has been reported in other cells (see Schmidt,'37a). Neither it nor any activity of the homogeneous cytoplasmic matrix which may exist can be considered as peculiar to the neural tissues.

There are, however, two structures within the cytoplasm which are more characteristic of these cells. They are the filamentous mitochondria which occur in other columnar cells, and the fibrils which are forerunners of the definitive neurofibrils.

Cowdry ('14), Meves ('08), Hoven ('10) and others have described the filamentous mitochondria in the cells of the neural tube of chick embryos at the ages studied here. They are arranged with their long axes roughly parallel to those of the cells and are not visible in unstained preparations. Their lipoid content and Giroud's description ('28) of birefringence in similar mitochondria in the gut wall of Ascaris make it probable that they contribute to the optical activity of these cells.

Cowdry also observed delicate neurofibrils developing in the neural tube from the 15 somite stage. The arrangement of these fibrils in the embryonic cells and their optical activity in the adult neuron suggests that they would increase the birefringence of the tube as they develop. Such an increase does not occur, however, and if they contribute to the birefringence of the cells it is only slightly.

The cytoplasm of the differentiated nerve cell also is optically active in both perikaryon (Chinn,'38) and axoplasm (Bear, Schmitt and Young,'37b). In both it is due to the contained protein neurofibrils and does not show the metatropic reaction. This further argues against the fibrils' playing the major role in the activity of the embryonic nerve cell, the cytoplasm of which shows the metatropic reaction in some instances.

It is not possible, therefore, to designate the particular structures within the embryonic nerve cell which are responsible for its birefringence, apart from the nuclear and plasma membranes. The latter are certainly important.

Although the optical activity of the developing neural structure is the resultant of the birefringence of several independent parts of the cell, the total activity can be considered as a unit, and the submicroscopic structure responsible for it can be discussed.

Considered in this manner, the form birefringence of the neural folds and tube is produced by that part of their protein constituents which is regularly arranged as long molecules with the principal axes paralleling the length of the cell or the surface of the membranes. These molecules are arranged in sheets in the membranes or as submicroscopic filaments in the protoplasm, but in both the resultant groups or micelles are anisodiametric and their refractive indexes average about 1.57. The slow component of this form bire-

fringence parallels the long axis of these micelles.

Lipoid molecules are associated with these protein molecules in such a way that their long axes are generally perpendicular to the micelles. These lipoid molecules are intrinsically birefringent and thus differ from the protein. Moreover, the slow component of the intrinsic birefringence parallels the long axis of the lipoid molecule and is at right angles to that of the form birefringence.

This submicroscopic arrangement of protein and lipoid is common in living matter, and the forces maintaining it have been widely studied (see Frey-Wyssling, '38). The existence of a dipolar force (heteropolar cohesive force) in the embryonic neural tissue is suggested by the effects of dehydrating agents, and the importance of pH variations is indicated by the stability of the lipoid components in fixatives containing acetic acid.

The loss of intrinsic birefringence with cell death demonstrates the greater lability of the lipoid than of the protein constituents. The work of Lepeschkin ('37) suggests that this is due to loss of orientation of the lipoid molecules rather than to their chemical destruction.

The subsequent development of this submicroscopic structure into that of adult nerve cells deserves further study. The changes at the cell surface with sheath formation are especially promising.

Unless the autonomous neural fold movements are due to

active cell migration and growth, as Gurwitsch ('14) believes, they depend upon intracellular changes and ultimately upon the ultrastructure of the folds. Glaser ('14) showed in the Amphibia that the water content of the neural structures during fold closure is higher than that of the remainder of the embryo and found no evidence of cellular multiplication or migration at this stage. The role he ascribed to water imbibition was upheld by Giersberg ('24), who prevented autonomous movements of the neural plate by growing amphibian embryos in sugar solutions.

Glaser ('16) also described a higher water content in the inner half of the folding neural plate of Cryptobranchus on the basis of differences in nuclear size. Further evidence of a differential water content is lacking, however.

The imbibition gradient which Glaser postulated across the wall of the neural plate could arise as the result of either of two circumstances: a differential permeability of inner and outer surfaces, or a differential distribution of structural elements, probably submicroscopic, across its width.

Heidenhain (quoted by Hoven, '10) has described microscopic differences between internal and external limiting membranes, but no differences in the optical activity were noted in this study. Moreover, the submicroscopic structure of the neural tube wall is such that there might be a gradient of imbibition across its width.

The results of dehydration by glycerine and salt solutions indicate that such an imbibition gradient exists and that it is capable of reacting differentially to the reverse process of dehydration. This gradient could exist only if the wall has a structural organization, and this organization is shown by the ultrastructure responsible for its birefringence. It is not possible to state definitely the constituent responsible for this gradient, but its disappearance with death suggests that the oriented lipoids play a considerable part.

The failure of the walls of the closed neural tube to spread during dehydration after the roof has been opened could be due to the loss of some previous differences in permeability at the tube surfaces. It seems more probable, however, that it is due to a loss of the imbibition gradient across the wall of the tube.

SUMMARY

1. The neural tube of surviving chick embryos shows measurable birefringence, positive with respect to the long axis of the cells in its walls. It has both form and intrinsic birefringence, largely in the nuclear and plasma membranes. The cytoplasm is also active and shows the metatropic reaction. Both protein and lipoid constituents therefore contribute to the activity of the tube.

2. Optical activity of the same sort is seen in the neural plate observed from the side, and in fold closure this is not changed. Some activity is also present in the plate as viewed from the surface.

3. The primitive streak area also shows optical activity, but this may not be due to the ectoderm. Other non-neural structures are birefringent, including somites, notochord, epithelial folds, and non-cellular membranes. All except the last show the metatropic reaction.

4. Dehydration of the embryo by concentrated salt and glycerine solutions causes a separation of the approximated and closing neural folds but not of the walls of the tube. This suggests that fold closure is the result of a differential imbibition of water by the neural plate.

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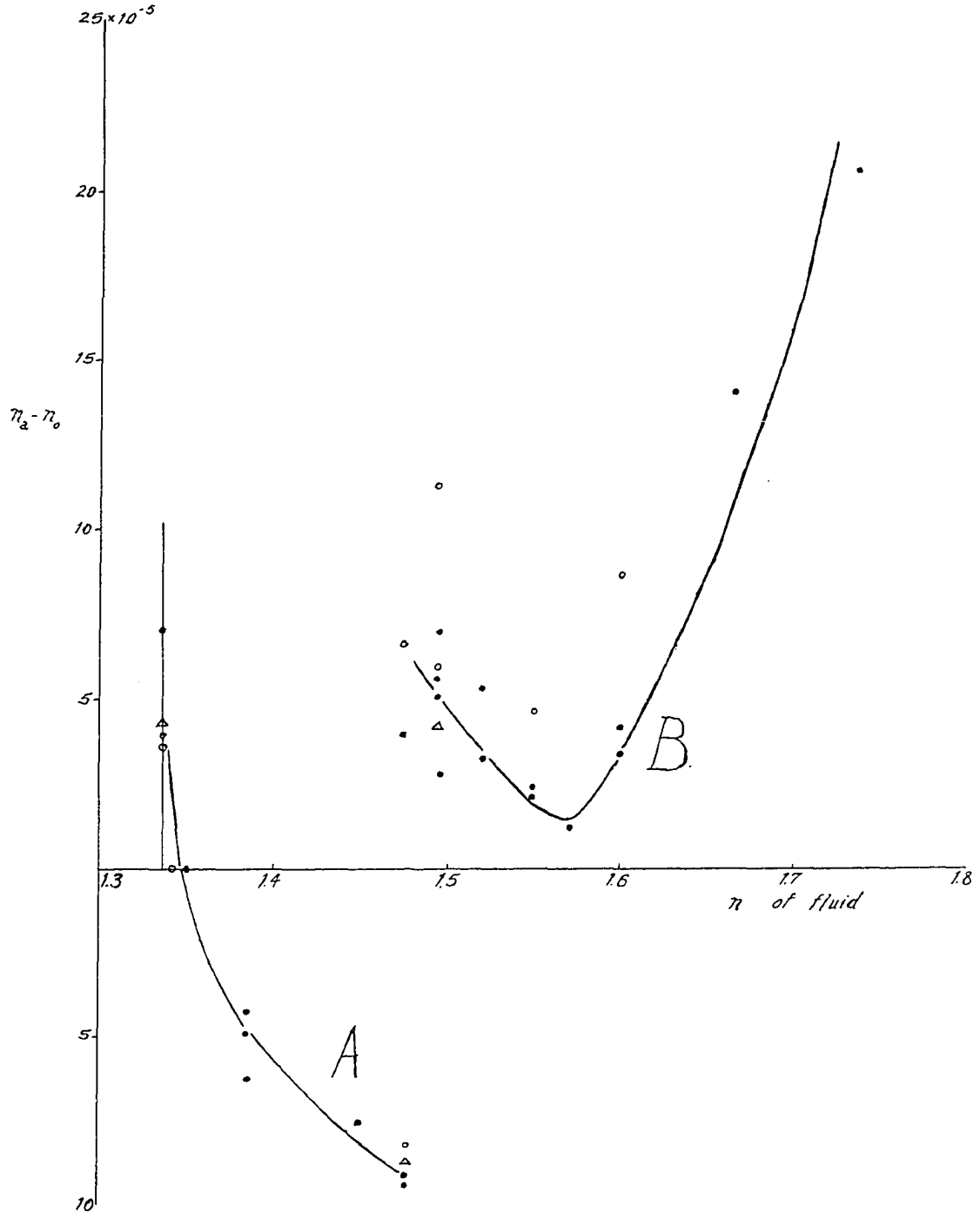

Fig. 1 Imbibition curves of neural tubes. A., unextracted tissue (in glycerine); B., lipoid-extracted tissue; ° , determinations on embryos of 3 to 4 somites; • , determinations on embryos of 8 to 14 somites; △, average values for somite 2 of Table 3, page 16.

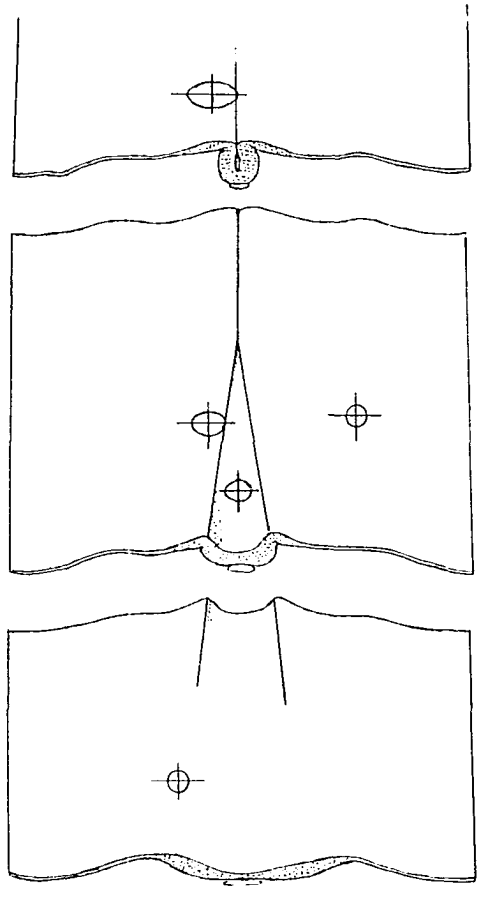

Fig. 2 Diagram of neural fold region of chick embryo. Superimposed index ellipses indicate optical properties in the living state.

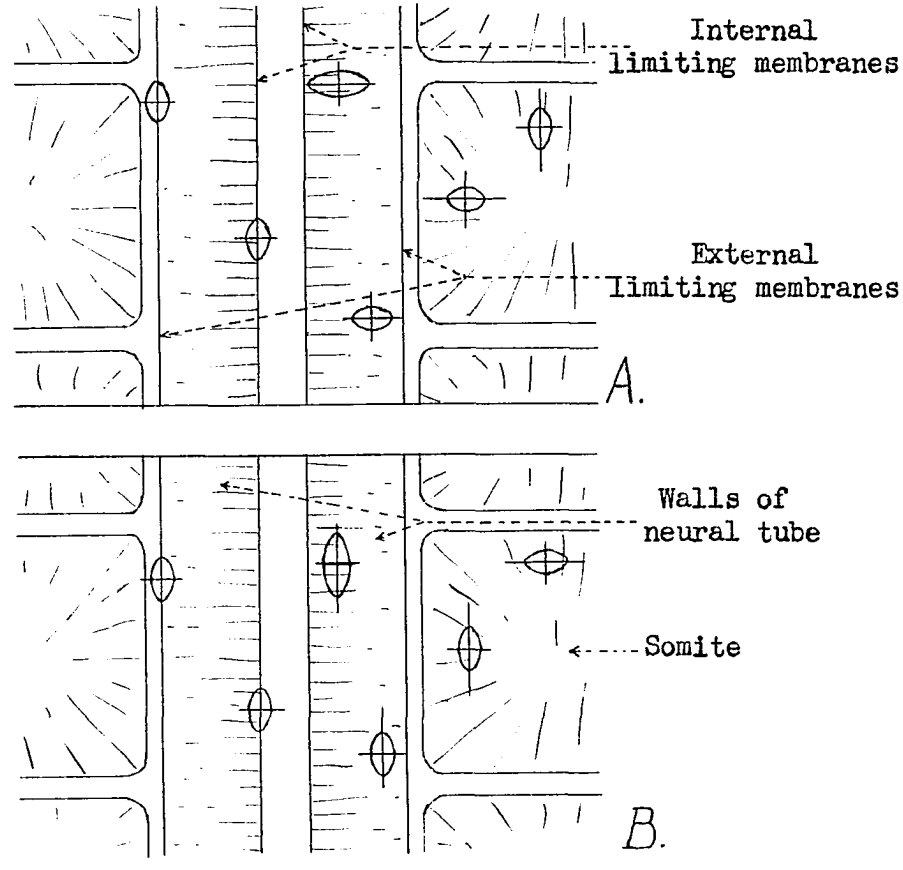

Fig. 3 Diagram of neural tube seen from above. A., optical properties in living and lipoid-extracted embryos, i.e., form birefringence; B., optical properties in concentrated glycerine, i.e., intrinsic birefringence.

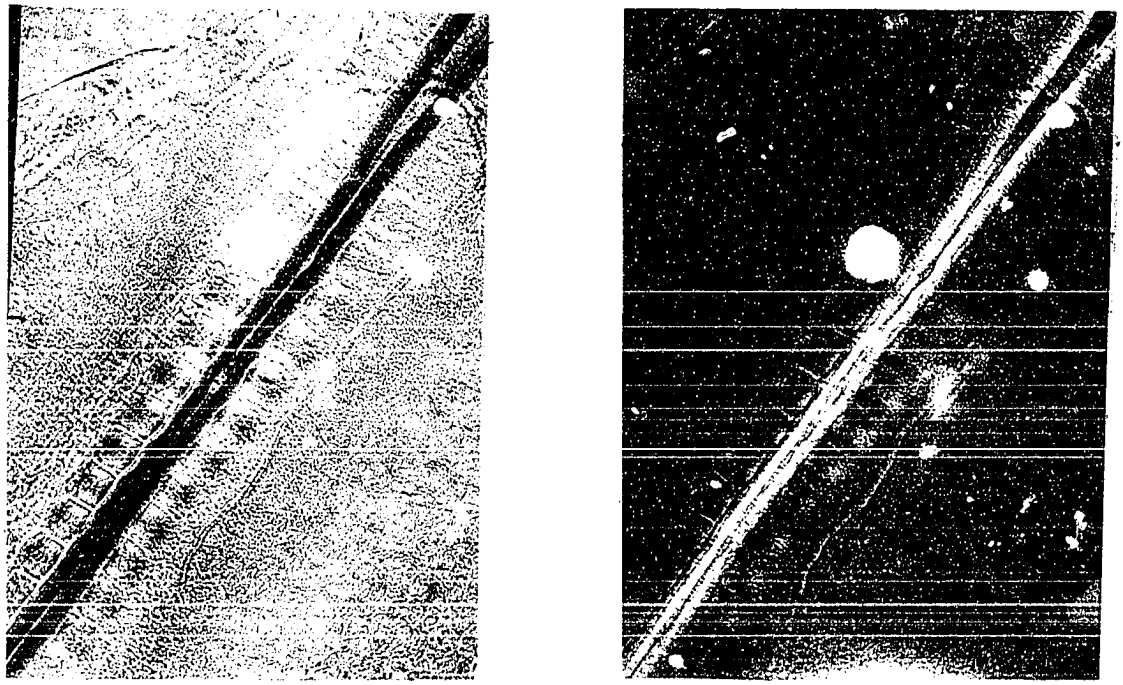

Fig. 4.-- Spinal cord of embryo in canada balsam. Prisms crossed and objective used as compensator; in additive position (to cord) right; subtractive, left.

Fig. 5.-- High power view of spinal cord with objective in additive position.