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COMMUNICATIONS

THE FORMATION OF ROSETTES IN THE RAT RETINA

BY

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INTRODUCTION.

Several conditions of the eye are known to be associated with the production of rosettes in the retina. Most, if not all, of these seem to be formed by complicated foldings of the outer nuclear layer so that the lumen of the rosette is bounded by the external limiting membrane.

Probably retinal rosettes are most often encountered in the tumour known as glioma retinae. As the name implies, this tumour was at one time thought to be composed of glial cells and therefore to be of ectodermal origin. Flexner (1891) was the first to show definitely that the growth was really produced from the outer layers of the retina and to suggest the name neuro-epithelioma for this type of tumour. He believed that the processes which are sometimes to be seen protruding into the lumina of the rosettes are really undeveloped rods and cones. Wintersteiner (1897) later enlarged on this conception in a detailed discussion of the condition.

Rosettes have also been found in the folded retinae of microphthalmic eyes and occasionally at the edges of colobomata.

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In all these cases rosettes are only to be found in eyes which, owing to some defect in development, are markedly abnormal. However, Lindenfeld (1913) and Goldstein and Wexlar (1931) describe rosettes in the retinae of the otherwise normal eyes of human foetuses. These foetuses were treated with large doses of X-rays during the second month of pregnancy. The irradiation was applied in order to terminate the pregnancy, but usually abortion did not occur until the fifth to the seventh month. Lindfeld's two cases had normally developed eyes and four out of the five cases described by Goldstein and Wexlar were also normal, though in the fifth case the foetal cleft was still present. All seven foetuses showed rosette formation in the retina. Pagenstecher (1916) was able to demonstrate rosettes in the retinae of rabbit embryos after irradiation by X-rays *in utero*. Rosettes have never been found in the retinae of normally developed adult eyes.

It would seem, therefore, that the natural production of rosettes is dependent on some abnormality in the development of the retina and it is also possible that X-ray treatment at a time when the eye is still in an embryonic condition upsets the normal development of the retina in some way and that the appearance of rosettes is a reflection of this disturbance.

I.—The Formation of Rosettes in Tissue Cultures of the Retina

The material to be discussed in the first section of the present paper consists of tissue cultures of the retina. Pieces of undeveloped retina from the chick, rat and human can all be cultivated *in vitro* and in all cases typical rosettes are produced. The only way in which these rosettes differ from those which appear *in vivo* is that the former are usually better formed and nearly always contain well-developed and easily recognizable rods and cones. It will be seen from the experiments described below that there is no doubt that the rosettes which appear during artificial cultivation are produced by involutions of the neuro-epithelial layers of the retina as described by Flexner and Wintersteiner.

The results presented here were obtained from tissue cultures of the rat retina. In the first series of experiments the material consisted of pieces of isolated retina from young rats cultivated by the hanging drop method, while in the second, whole embryonic eyes from rat foetuses were used. In this series the tube technique was employed.

THE ISOLATED RETINA.

Technique. The rat eye is by no means fully developed at birth (Fig. 1), in fact, the retina does not become adult in appearance until the animal is about 17 days old (Fig. 2).

For the present experiments young rats were killed three to five days after birth and the eyes enucleated. Since the eyelids are still fused at this stage, it is quite easy to dissect out the bulb aseptically. One eye was fixed in Zenker's solution as a control while the retina was removed aseptically from the other. The

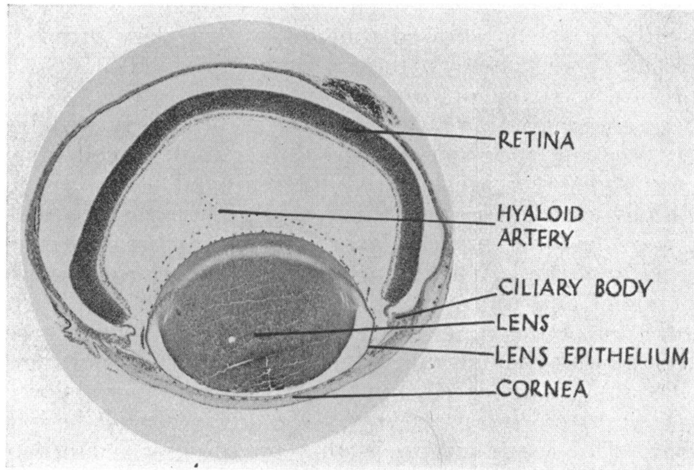

FIG. 1.

Section through the rat eye at birth × 20. (Zenker; Haematoxylin and Eosin.) (Phot. R. J. L.)

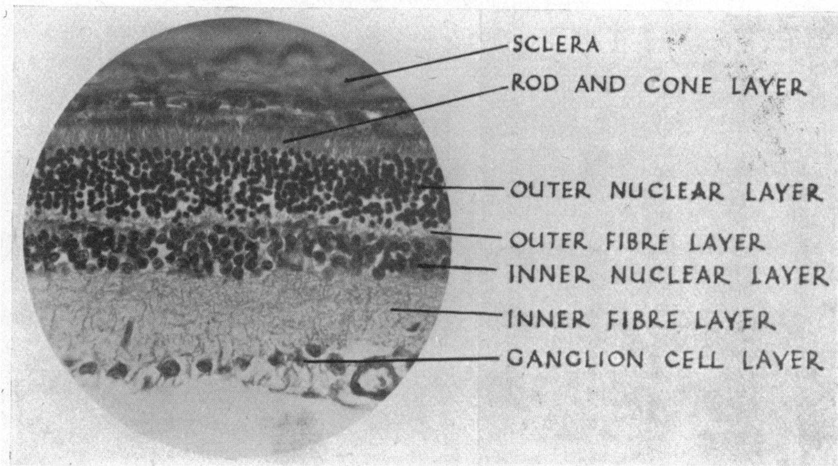

FIG. 2.

Section through the rat retina 17 days after birth × 260. (Zenker; Masson's triple stain.) (Phot. R. J. L.)

retina was well washed in an aseptic saline extract of chick embryo and cut into pieces of approximately 1 sq. mm. Each piece was spread on the surface of a clot made by mixing one drop of chick plasma and one drop of embryo extract on a coverslip. Each coverslip was inverted over a hollow ground slide, sealed down with wax and incubated at 37° C.

Chick media were used for convenience but preliminary experiments with rat media showed that these gave very little, if any, better results even when rat tissues were cultivated.

Condition of retina at time of explantation. Fig. 3 shows a section of the rat retina three days after birth. It will be seen that the layer of optic nerve fibres, the ganglion cell layer and the inner fibre layer are already differentiated. The rest of the retina is composed of a very thick nuclear layer which will later divide into the inner nuclear layer, the outer fibre layer and the outer nuclear layer. At this stage, this layer is largely composed of oval nuclei and there is, as yet, no indication of the coming differentiation of the inner nuclear layer. Numerous mitoses are to be seen among the outermost cells. The external limiting membrane has already appeared, but there is no sign of rods or cones.

Observations on living cultures. One advantage of the hanging drop method of tissue culture is that microscopic examination is possible while the explants are still under cultivation. If the coverslip and the clot are not too thick it is quite easy to use magnifications up to $\times$ 300.

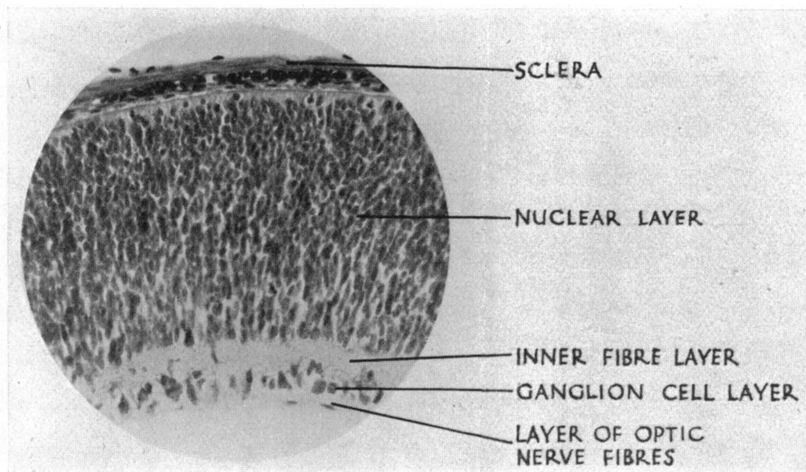

FIG. 3.

Section through the rat retina 3 days after birth $\times$ 180.
(Zenker; Haematoxylin and Eosin.) (Photo R. J. L.)

During cultivation *in vitro* the explants may increase in size, but examination under the microscope shows that this is due to the mesodermal elements of the retina and that the nervous elements have not increased.

After two days incubation rosettes may already be formed in the centre of the explant. These are usually rather large in size and their lumina can be seen to be bounded by the external limiting

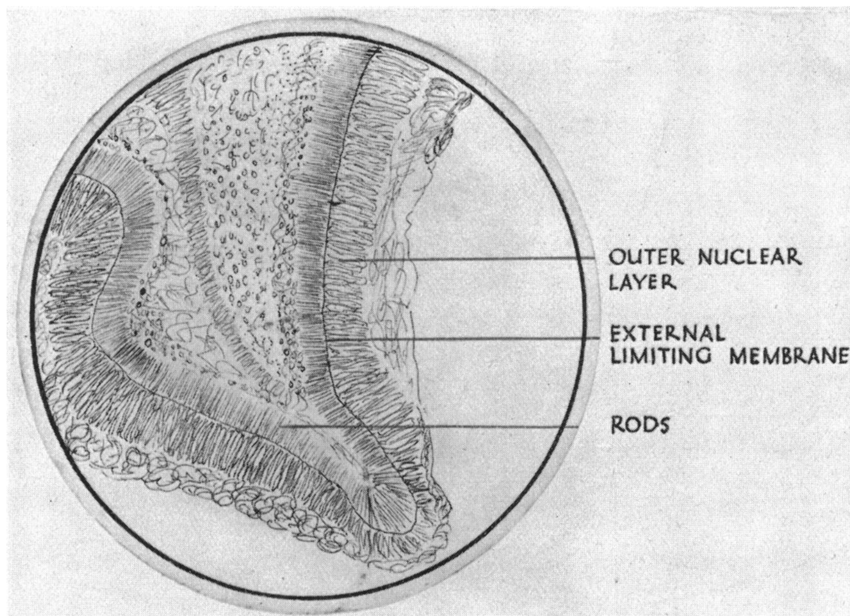

FIG. 4.

Living culture of the retina of a 3-day rat after 5 days cultivation *in vitro*. Part of a rosette is shown. (Del. K.T.)

membrane. At this stage, short rods can be seen protruding through the membrane into the lumen of the rosette. The columnar arrangement of the nuclear layer is very obvious in living cultures. After five days incubation the rosettes are much more numerous and a good deal smaller in size. At this stage, the formation of small new rosettes by nipping off from already existing ones can be demonstrated by observations on a suitable field over a period of several hours during incubation on a hot microscope stage. The rods are now much longer and easily recognisable (Fig. 4). After six days, further incubation does not produce further differentiation although the cultures may remain healthy for another week. The glial and mesodermal elements now increase, apparently at the

expense of the nervous elements which gradually disappear. Transferring the explant to a fresh clot at intervals encourages this growth of mesoderm.

Histological development of the explants. After microscopic examination of the living preparations the explants were fixed in Zenker's solution, sectioned and stained either with haematoxylin and eosin or with Mallory's phosphotungstic acid haematoxylin.

The general appearance of a culture of a three day old rat retina after five days growth *in vitro* can be seen in Fig. 5. It

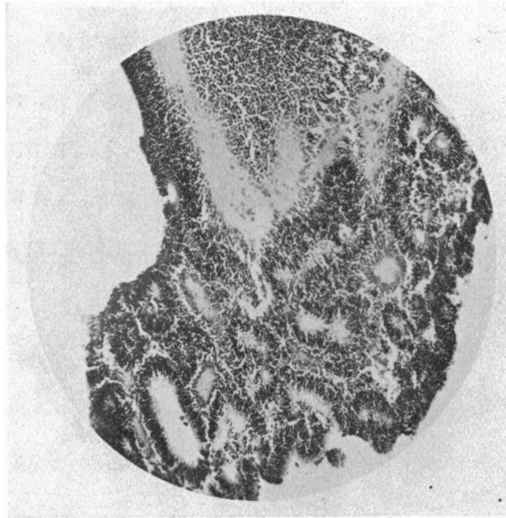

FIG. 5.

Section through a culture of the retina of a 3-day rat after 5 days cultivation *in vitro*. × 80. The culture is full of rosettes. (Zenker; Haematoxylin and Eosin.) (Phot. R. J. L.)

is full of well-marked rosettes of various sizes which are formed by the outer nuclear layer with the rods on the inside pointing into the lumen. Fig. 6 is a high power view of one rosette in a similar culture. In this specimen the external limiting membrane, with the rods protruding through it, can be clearly seen. The inner nuclear layer has divided off and its nuclei have become round, while the outer fibre layer is just beginning to appear, but is still very small. Mitoses seem to be absent from these cultures and this confirms the observations made on the living preparations, that there is no actual increase in size of the neuro-epithelium during cultivation *in vitro*. Detwiler (1932) has also shown that

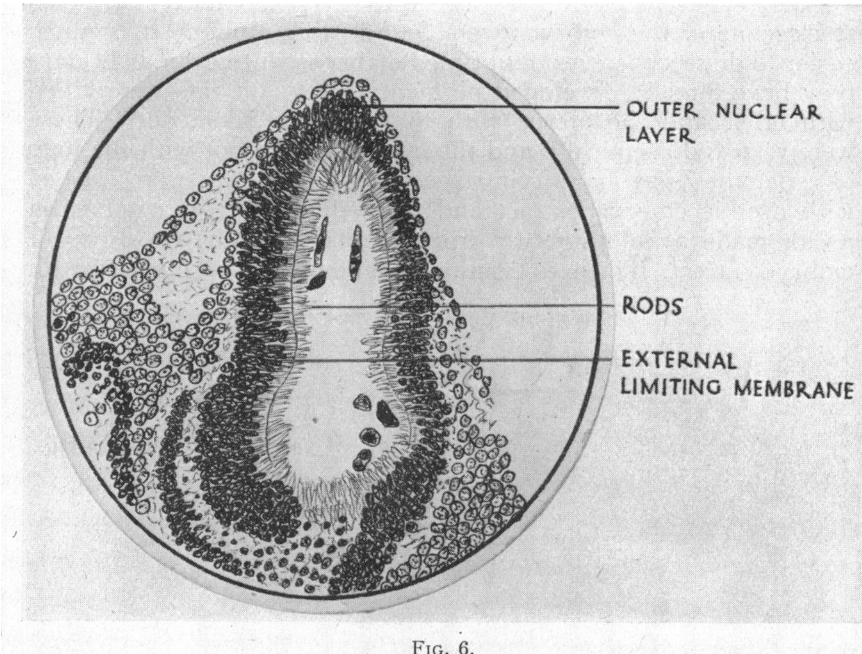

FIG. 6.

Section through a culture of the retina of a 3-day rat after 5 days cultivation *in vitro*. One rosette is shown. (Zenker; Mallory's phosphotungstic acid haematoxylin.) (Del. K. T.)

mitoses have ceased to occur in the outer part of the retina at this stage during development *in vivo*.

The stage of development of a piece of retina from a three day old rat after five days cultivation *in vitro* is very little behind that of the retina of an eight day old rat, so that during these first five days differentiation *in vitro* must proceed at nearly the normal rate.

ISOLATED EMBRYONIC EYES.

Technique. Strangeways and Fell (1926) succeeded in growing the embryonic eye of the chick, and their paper gives a very full account of the development of these eyes during 17 days cultivation *in vitro*. By using the same technique it is also possible to grow embryonic rat eyes *in vitro* for periods up to 21 days and the general developmental picture is very similar in both cases, particularly if the rat's eyes used are from embryos of approximately 14 days or less. The experiments to be described below fall into two groups—that in which the eyes were taken from embryos about 14 days old, and that in which the embryos were about 17 days old.

Pied rats were used, but since these were bred from a mixed stock some of the embryos were found to be albino. It is much easier to dissect the eyes out of pied embryos since even at 14 days they have already developed pigment. Pregnant rats were killed with ether and the uterus with the embryos taken out. These were removed aseptically and the eyes dissected out with the help of a pair of Zeiss magnifying spectacles. The eyes were washed with aseptic embryo extract and planted in centrifuge tubes on a clot made by mixing two drops of plasma with two drops of embryo extract. The eyes from the older embryos were placed with

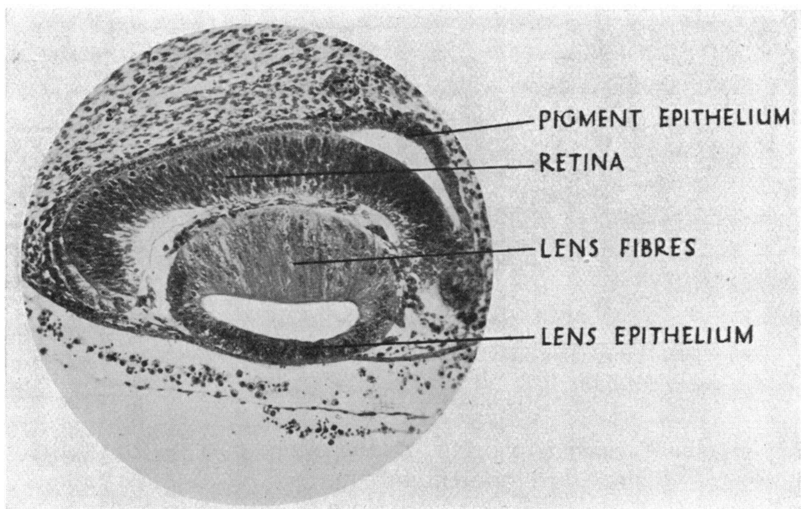

FIG. 7.

Section through the eye of a 14-day rat embryo. ×120.
(Bouin ; Haematoxylin and Eosin.) (Phot. R. J. L.)

the cornea uppermost and the back of the eyeball in contact with the clot. Chick media were used throughout. The centrifuge tubes were corked up and incubated at 37° C. The eyes were transferred on to a fresh clot every 72 hours. One embryo from each litter was fixed in Bouin's solution as a control.

Condition of eyes at time of explantation. 14-day embryos. Fig. 7 illustrates the stage of development of the eye of the rat embryo at 14 days. It will be seen that, although the lens is completely separated from the covering ectoderm, it is by no means fully developed. The lens epithelium in front is about three cells thick and is not yet in contact with the lens fibres which are already forming. The retina is still composed of undifferentiated epithelium, though there is an indication of the formation of the

layer of optic nerve fibres—the first layer to appear during the differentiation of the retina. The ciliary body is, as yet, unformed. There is no sclera in these eyes and the bulb, when dissected out, is bounded only by the pigment epithelium which already contains pigment in those embryos which will become pied rats. (The eye illustrated is from an albino embryo.) In many cases fragments of mesoderm are brought away with the bulb.

17-*day embryos*. In Fig. 8 a section through the eye of a 17 day rat embryo can be seen. At this stage the lens is almost fully

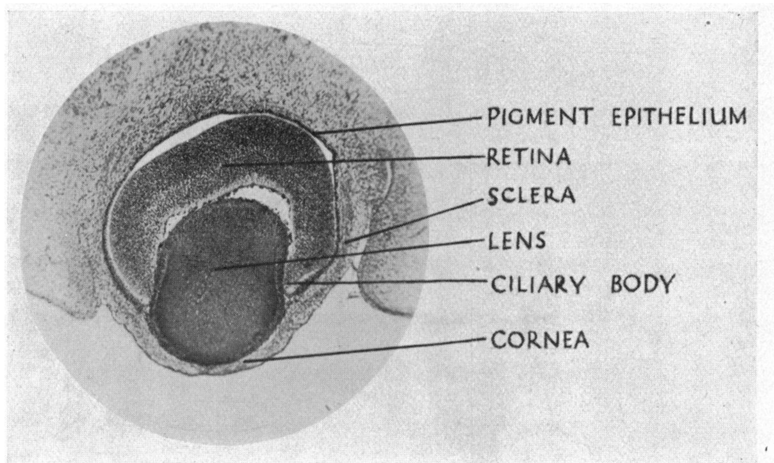

FIG. 8.

Section through the eye of a 17-day rat embryo. ×44.
(Bouin; Haematoxylin and Eosin.) (Phot. R. J. L.)

developed, the lens epithelium is only one cell thick and in contact with the fibres. In the retina, the first indication of the differentiation of the ganglion cell layer can be recognized and the layer of optic nerve fibres is already present. The ciliary body is already pigmented, and can be seen as a dark ring in fresh preparations. At this stage the sclera can be dissected out with the bulb.

Observations on living cultures. Microscopic observation of these cultures during incubation is not possible.

14-*day embryos*. After about six days cultivation *in vitro* the explants can be seen to have spread out on the clot so that they take the form of a halo of thin tissue round a central thick area which contains the black pigment. Examination of sections of these cultures under the microscope shows that the thin sheet of tissue is the retina while the central area contains the lens, pigment epithelium and any mesoderm that may have been dissec-

ted out with the bulb (see Fig. 9). After a further three days incubation the lens becomes necrotic and shows up as an opaque white spot in the centre of the explant.

17-*day embryos*. Eyes at this age are too big for satisfactory cultivation *in vitro* and only about 25 per cent. survive. Even in those cultures which do survive it is usually only that part of the eye which is in direct contact with the clot that remains healthy. The lens always becomes necrotic during the first three

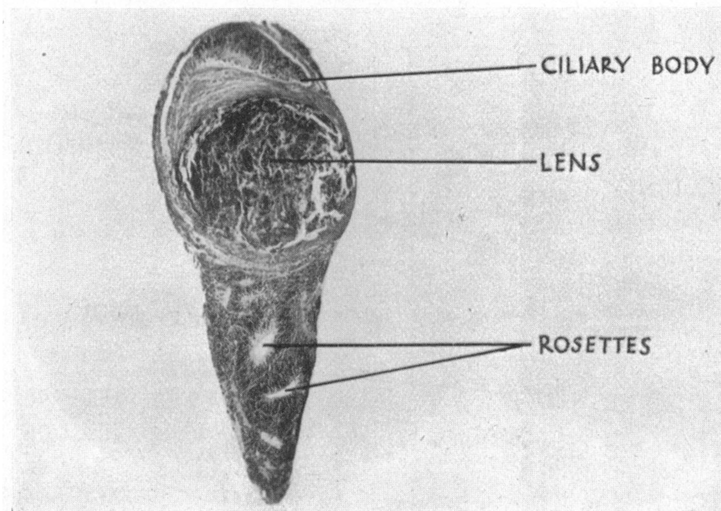

FIG. 9.

Section through a culture of the eye of a 14-day rat embryo after 15 days cultivation *in vitro* X60. The retina has burst out of the bulb and has formed rosettes. (Zenker; Haematoxylin and Eosin.) (Phot. R. J. L.)

days of incubation. The explants do not appear to increase in size at all and always remain spherical, so that apparently the intra-ocular pressure is not appreciably altered by this treatment.

Histological development of explants. At varying times after explantation the cultures were carefully dissected out of the clot with the help of a pair of Zeiss magnifying spectacles, fixed in Zenker's solution and stained with haematoxylin and eosin or with Mallory's phosphotungstic acid haematoxylin.

Explants from 14-*day embryos*. Fig. 9 shows a section of the eye from a rat embryo of 14 days after 15 days cultivation *in vitro*. It will be seen that there has been considerable differentiation though there has been very little, if any, increase in size. The lens has become necrotic though healthy lens epithelium can be

recognized in places. The ciliary body has been formed and is already pigmented; this and the pigment epithelium remain in contact with the lens and have in general retained their normal appearance and position. Most of the retina, however, has burst out of the bulb and can be seen lying outside the pigment epithelium. It is full of rosettes, many of which contain a definite lumen bounded by the external limiting membrane. Under high magnifications, young rods can be seen protruding into the rosettes. The retina is not yet fully differentiated, but the ganglion cell layer and the inner fibre layer can be recognized. These cultures have reached a stage of development corresponding to that of the eye of the rat six to eight days after birth. In this case, therefore, differentiation seem to proceed at nearly the normal rate, since the actual age of the artificially cultured eyes is 29 days (14 days *in vivo* plus 15 days *in vitro*) which is equivalent to seven days after birth (the gestation period of the rat is 22 days).

Explants from 17-day embryos. The section illustrated in Figs. 10 and 11 is from a culture after 21 days *in vitro*. It has not been found possible to keep these cultures alive any longer than this. It will be seen that the eye has retained its normal shape. The bulb is bounded on the outside by the sclera which is lined by the pigment epithelium. Inside this lies the retina which has,

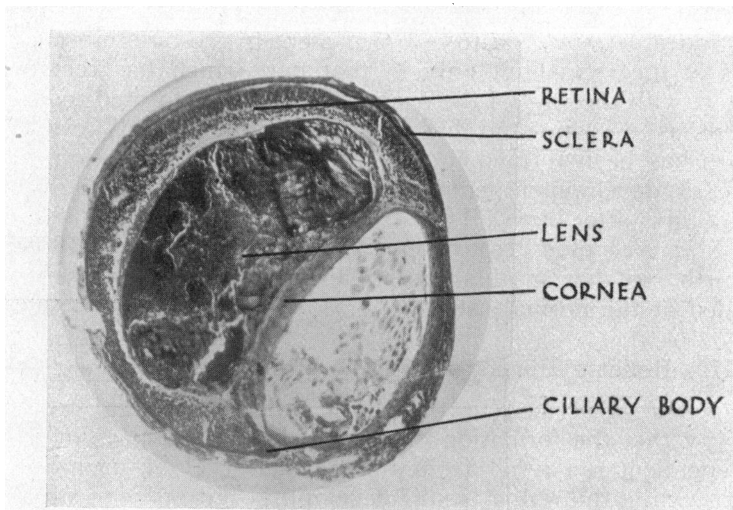

FIG. 10.

Section through a culture of the eye of a 17-day rat embryo after 21 days cultivation *in vitro*. × 64. The retina has remained in its normal position and has formed only one or two rosettes at the site of removal of the optic nerve. (Zenker; Mallory's phosphotungstic acid haematoxylin.) (Phot. R. J. L.)

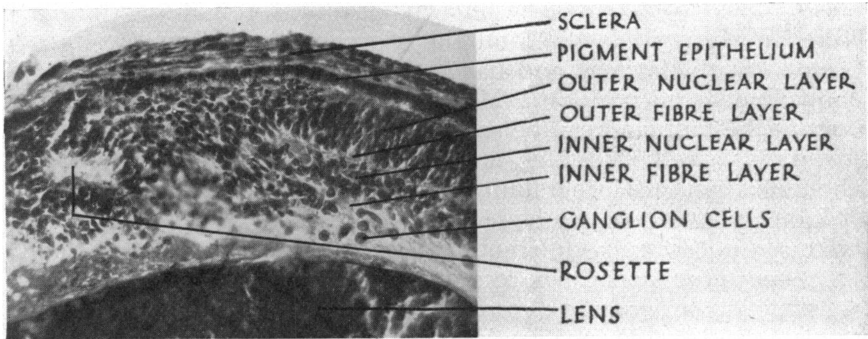

FIG. 11.

Part of the section illustrated in Fig.10 X220. The formation of a few rosettes at the posterior pole can be seen. (Phot. R. J. L.)

for the most part, continued to differentiate into layers in an orderly manner. A few small rosettes are to be found at the posterior pole where the optic nerve was cut away during removal of the eye from the embryo. All the layers of the retina are present, though the ganglion cell layer is only represented by a few scattered cells. Rods are present and can be seen under high magnification both within the rosettes and along the outer surface of the retina; unfortunately they are not distinguishable in the section illustrated. Mitoses are present in the outermost layer of cells of the retina but only in that part which has remained in contact with the wall of the eyeball and has not formed rosettes. The lens is necrotic and has lost its epithelium. A cyst-like structure has been formed in the cornea. This preparation is at a stage of development equivalent to that of a normal eye at about 12 days after birth. The actual age of the culture is 38 days (17 days *in vivo* plus 21 days *in vitro*), corresponding to 16 days after birth, so that in this case differentiation *in vitro* has not proceeded at the normal rate.

II.—Rosette Formation in the Rat Retina *in vivo*

The results described in Part 1 of this paper suggested the possibility that the formation of rosettes by the retina is in some way dependent on a fall from the normal level of intra-ocular pressure, while the retina is still developing. In order to procure further evidence on this point the eyes of young rats were trephined at about five days after birth and the retinae of these eyes examined histologically after several days further growth *in vitro*.

Technique. It was found convenient to operate on all the members of one litter at the same time. One eye of each young rat was treated while the other was left untouched as a control.

Before the operations the litter was separated from the mother who was lightly anaesthetised with ether when the operations were over. She was then returned to the cage with the operated young rats and they were all allowed to recover together. It was found that when treated in this way the mother would continue to nurse her litter without any trouble.

Each young rat was anaesthetised with ether and the eyelids separated with a Graefe knife. A stitch of human hair was put through each lid and used to hold them apart. The eyeball was then gently rotated with a pair of fine curved forceps and the sclera cut with a 1 mm. trephine as far posterior to the corneo-sclerotic junction as possible. The eyelids were then sewn together with one stitch of human hair. Later it was found that it was not necessary to trephine into the posterior chamber, the eye illustrated in Fig. 12 was trephined just anterior to the corneo-sclerotic junction.

The animals stood the operation well and it was found that, in those that were kept long enough, both the operated and the control eyelids opened at approximately the same time. The rats were killed at varying times after the operation and the eyes were removed, fixed in Zenker's solution, sectioned and stained with haematoxylin and eosin or with Mallory's phosphotungstic acid haematoxylin.

Results. The operated eye is always smaller than the control and the longer the animal is allowed to live after the operation the greater is the difference in size between the two eyes. Sometimes the eye may appear slightly collapsed but mostly the pressure is good and the site of the trephine hole is often very difficult to find.

Under the microscope the retina is seen to be thrown into folds and to remain attached to the wall of the eye in a few places only. It is full of rosettes formed by the inward folding of the neuro-epithelial layer (Fig. 12). Rods may be seen protruding into the lumina of these rosettes, and in some cases, the pigment epithelium is also to be found in contact with the rods in the rosettes. The development of the retina seems to continue at a normal pace, at any rate for the first week after the operation. Except for the retina, the microscopic structure of these eyes appears to be absolutely normal.

Concluding remarks. There is no doubt that the rosettes which appear in the retina, both in tissue cultures and as a result of trephining the growing eye, are formed from the neuro-epithelial layers as described by Flexner and Wintersteiner. In fact, it seems probable from a careful study of the literature that in all cases where rosettes are formed, they are produced from this part of the retina.

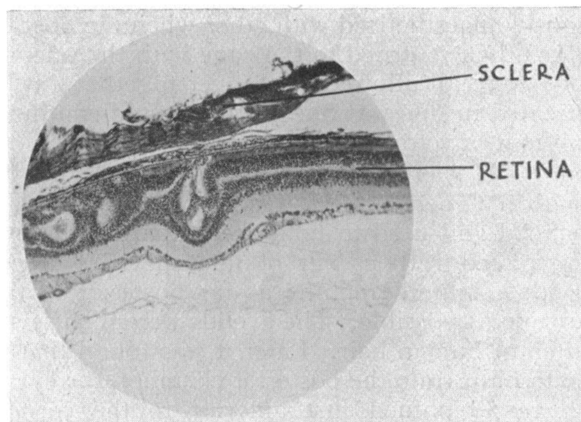

FIG. 12.

Section through the eye of a rat ×50. This eye was trephined 6 days after birth and the rat killed 38 days after operation. The retina is full of rosettes. (Zenker; Mallory's phosphotungstic acid haematoxylin.) (Phot. R. J. L.)

The formation of rosettes appears to be essentially a feature of the developing retina and probably never occurs in one that is fully differentiated. In tissue culture, rosettes cannot be obtained in retinae from rats of more than about 15 days old.

It has been suggested that rosettes appear as a result of some disturbance in development at a stage at which the neuro-epithelial part of the retina is particularly active in growth. As far as my observations go, however, it seems that there is no one stage of development which is especially favourable to the production of rosettes. In tissue cultures, in which the retina is isolated, or subsequently becomes isolated, rosettes can be obtained with equal ease at any stage between that of a 12-day rat embryo, where the retina is nothing but a layer of undifferentiated epithelium, and that of a rat 11 days after birth where, with the exception of the rod and cone layer, the retina is completely developed.

When rosettes are formed in a retina which is situated within the eye it is easy to see that an abnormal growth of the neuro-epithelial layers might cause *inward* folding of the retina, for since the expanded tissue must be accommodated somewhere, it is probably easier for it to penetrate the inner layers of the retina than the hard fibrous layers of the choroid and sclera. In tissue cultures, on the other hand, one might expect the outer layers of the retina to bulge *outwards* since here there is no obstacle to expansion in this direction. The phenomenon of rosette formation by inward folding under these conditions could be explained

if we assume a differential stretching or shrinkage between the external limiting membrane and the neuro-epithelial layers internal to it. That there is normally some difference in tension between the outer and inner parts, even in the adult retina, can be shown by removing it from the eye. If the tissues are still living, it will be found that the retina always curls up into a roll so that the rod and cone layer is on the inside.

A serious objection to the view that rosettes are produced by an overgrowth of the neuro-epithelium is that, in any case in tissue cultures, mitoses have not been observed in the cells immediately surrounding the rosettes, although they are frequent in this part of the retina in the normally developing eye. The only occasion on which I have been able to find mitoses in tissue cultures of the retina is in the preparation illustrated in Figs. 10 and 11, and these occur only in the part of the retina which has not formed rosettes.

Under the conditions described in this paper there certainly seems to be a definite relation between the maintenance of a normal intra-ocular pressure and the appearance of retinal rosettes. In all the cases with which we have been dealing here the fact of a lowering or an absence of intra-ocular pressure also involves the dissociation of the retina from the wall of the eye to which it is normally attached during development. It is possible that here we have another instance of the well known effect of connective tissue in inducing an orderly growth of the epithelial tissues in contact with it. We must, however, recognize that normally the retina is not in direct contact with the connective tissue of the wall of the eyeball but with the pigment epithelium and that in all these experiments this tissue remains in its normal position and does not appear to lose its capacity for orderly development. Strangeways and Fell, on the other hand, report that in their cultures of the embryonic chick eye the pigment epithelium may be found folded upon itself to form complicated tubules. These cultures, however, were prepared from eyes at a much earlier stage of development than those described here and any mesoderm which was dissected out with the bulb in the first instance was usually lost during the first change on to a new clot. In any case, however, if we assume that the production of rosettes during development of the retina is due to the loss of some orderly influence which is normally provided by the connective tissue, it seems that we must also assume that such an influence can be transmitted across the intervening pigment epithelium.

Summary

(1) The technique for making tissue cultures of the isolated undeveloped rat retina is described and the appearance of rosettes in these cultures reported.

(2) Whole embryonic rat eyes can also be cultivated *in vitro*. Rosettes are formed in the retinae of the younger eyes but not in those of the older ones.

(3) Retinal rosettes can be produced *in vivo* by trephining the undeveloped eyes of young rats soon after birth.

(4) The connection between a lowered intra-ocular pressure and the production of rosettes in the retina is discussed.

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"WHITE RINGS" IN THE CORNEA

BY

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GLASGOW

IN the *Trans. of the Ophthal. Soc. U.K.* for 1912,[1] George Coats described two cases "showing a small superficial opaque white ring in the cornea," and in the *Proc. Roy. Soc. Med.* for 1913,[2] he published a third case. As I have seen no further detailed reference to this condition, and as I have observed four or five cases which belong to the same category, I thought it might be of interest to give a brief description of them.

Coats' description of the lesion as seen with the loupe is so complete and precise, that even with the advantage of the higher magnification available with the slit-lamp there is not a great deal to add. In his first case, a man, aged 18 years, the cornea presented, a little below its centre, a white ring, measuring about