Development of embryonic rat eyes in organ culture

I. Effect of glutamine on normal and abnormal development in a chemically defined medium

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Previous studies on the differentiation of whole embryonic eyes in culture have been carried out mainly in the chick (Strangeways & Fell, 1926; Dorris, 1938; Harrison & Berry, 1959) and in one case in the rat (Tansley, 1933). Early postnatal intact eyes of rats and mice were cultured by Lucas & Trowell (1958), while more recently intact and trypsinized early eye rudiments (9 and 10 days) of mouse embryos have been grown in various modified organ culture systems (Muthukkaruppan, 1965). In all these experiments the media employed were either entirely biological or chemically defined media supplemented with biological media. Also, with the exception of the work done by Muthukkaruppan (1965), eye differentiation was well advanced at the start of the culture period.

The present study was designed to test the feasibility of observing early embryonic rat eye development in organ culture with a totally defined medium. The ultimate aim was to devise a system in which the effects of individual components of the medium on embryonic eye development could be more precisely determined. This report describes such a system as well as the degree of eye differentiation achieved under these \textit{in vitro} conditions.

\textbf{MATERIALS AND METHODS}

Pregnant rats of the Long-Evans strain, 3–4 months of age, were maintained on a stock diet to the 10th, 11th, or 12th days of gestation; the day of finding sperm in the vagina was considered as day zero. Embryos from animals at these stages of pregnancy provided material for organ culture.

The pregnant animals were killed by cervical dislocation after light ether anesthesia and the entire uterus was removed under sterile conditions and placed in dishes containing medium. In the case of day-10 embryos the fetal membranes were removed and the entire organism was cultured. Explants from day-11 embryos, however, consisted of the separated intact head, while those

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from day-12 embryos consisted of a sagittal section of the head. The average size of explanted tissues was approximately 1.5-2.0 mm.

The watch-glass/Petri dish complex described by Fell & Robison (1929) was used as the culture vessel. One ml of Waymouth’s chemically defined medium-MB 752/1 (Waymouth, 1959) was added to each watch-glass at the start of the culture period and a lens paper, prepared according to the method of Chen (1954), was floated on it. Four explants were then placed on each lens paper, and cultured at 37 °C.

The culture period was either 5 or 6 days and the medium was changed only on the third day of culture. At the end of the culture period all explants were fixed in Bouin’s solution, dehydrated in ethanol, infiltrated and embedded in paraffin, and serially sectioned at 7 μ. Hematoxylin and eosin were used to stain the sections which were then examined histologically.

RESULTS

1. Normal Histology

The normal histological appearance of the eye in the early stages of development in vivo (i.e. days 10, 11, 12) will be considered briefly.

In day-10 embryos the eye anlagen appear as two outpushings from the diencephalon, the optic vesicles, and are separated from the surface ectoderm by one or two layers of mesodermal cells. On day 11 the optic vesicles are almost in direct contact with the overlying surface ectoderm and lens placodes appear about 12 h later. The walls of the vesicles have thickened and appear somewhat flattened as they approach the surface ectoderm; each now exhibits a well-defined cavity. In day-12 embryos the optic vesicles invaginate and form optic cups which consist of an inner thick layer, the future neural retina, and an outer thin layer, the future pigmented layer of the retina. Pigment granules do not appear in the latter until 24 h later. A lens vesicle, still connected to the surface ectoderm, but no longer communicating with the exterior, is also present.

2. Response of 10-, 11-, and 12-day embryonic rat eyes cultured in Waymouth’s medium

Experiments were first undertaken to determine the most suitable stage for culture of the eye rudiment. When eyes from embryos at days 10, 11 and 12 were cultured in Waymouth’s medium at pH 7.8 with a gas phase of 95 % air/5 % CO₂, notable differences between the age-groups were seen. In day-10 embryos observed after 6 days of culture the gross shape of the embryos was not maintained, and histological examination revealed that organ rudiments, including the eye rudiments, present at the onset of culture were no longer identifiable as such. Various ‘ectodermal-like’ vesicles and mesodermal aggregations of undetermined origin were observed in their place.

After 6 days of culture, day-11 embryos underwent differentiation equivalent
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to 3 days of development in vivo. Although the rate of differentiation in vitro was considerably slower, the pattern of development was normal.

On the whole, explants from day-12 embryos consistently degenerated under these in vitro conditions. Various alterations in technique did not improve the results.

The above results showed that day-11 embryonic eye rudiments (cultured as intact heads) exhibited consistently superior eye differentiation under the culture conditions employed, compared with tissues from day-10 and day-12 embryos; for this reason only day-11 embryos were used as culture material in subsequent work.

3. Effect of culture conditions

A. General observations

After 6 days of culture in Waymouth’s medium at pH 7.8 in 95% air and 5% CO₂, the optic vesicles of day-11 embryos invaginated to form optic cups, and pigment granules were sometimes present in their outer layers (Plate 1, fig. A). The lens placode also formed and invaginated to give rise to the lens vesicle with well-differentiated lens fibers and a well-defined layer of cuboidal cells representing the anterior epithelium (Plate 1, fig. B). In a few cases evidence of corneal formation was present in that the lens had separated from the surface ectoderm and the latter had sealed over to form the future epithelial layer of the cornea. Occasionally mesenchymal cells could be observed between the anterior epithelium and the overlying surface ectoderm.

Although this degree of differentiation was observed in a majority of the eyes after 6 days in culture, several eyes exhibited early degenerative changes at this stage. For this reason most cultures were terminated after 5 days when differentiation was at a peak and degeneration was minimal (for details of time-study observations see §4).

Culture under various pH conditions showed that 11-day embryonic eye explants in medium below pH 7.0 or above pH 8.0 underwent complete necrosis. Generalized degeneration was also observed in explants cultured in gas phases of 95% O₂/5% CO₂ as well as in 95% N₂/5% CO₂. Further, the viability of the entire explant under standard conditions was greatly enhanced when it was oriented so that the crown of the head rested on the lens paper (i.e. in contact with the medium), as compared to the severed surface resting in contact with the medium.

B. Effect of glutamine

The above results established that lens induction occurred and differentiation continued normally, although slower than in vivo, in day-11 explants grown in Waymouth’s medium. The Waymouth’s medium used in these cultures contained glutamine at the time it was obtained, and the medium was stored until used. However, since Tritsch & Moore (1962) reported that glutamine decomposes rapidly in solution, a preparation of Waymouth’s medium was obtained
without glutamine and freshly prepared glutamine was added (according to the original formula, 350 mg/l) just prior to use.

Embryonic eye anlagen (day 11) cultured in Waymouth’s medium to which 350 mg/l freshly prepared glutamine was added showed striking changes from the previous cultures. All tissues, including mesenchyme, surface ectoderm, and brain, appeared healthier. Some areas of necrosis were still observed, but mitotic figures were more prevalent than before and the populations of cells showed less tendency toward degeneration.

Another difference was the marked increase in the size of the lenses under these culture conditions. The size of the lenses exceeded that observed in any previous experiments and was almost invariably accompanied by one or more morphological aberrations of development. Many of the large lenses exhibited swollen lens fibers and lens sutures located in abnormal positions (Plate 1, fig. C). In some cases, these lenses appeared to be composed of two or three individual masses which had fused to form a single unit (Plate 1, fig. D). Lens fibers were also observed herniating anteriorly in the position of the anterior epithelium. Other globular protrusions from the posterior or lateral surfaces of the lens invaded the cellular mass of the optic cup or perhaps the choroidal fissure. In the latter case, the anterior epithelium was usually normal.

Large lenses were at times accompanied by thickened retinae. The neural retina was often as many as ten cell-layers thick without showing any degenerative changes. Isolated masses of cells or diverticula which seemed to be derived from the neural layer were frequently observed lying between the neural and pigment epithelia (Plate 1, fig. E). Mitotic figures in significant numbers were observed in irregular distribution within the neural retina and were usually associated with fibrillar patches, making them resemble rosettes. The pigmented epithelium consisted of a normal layer of cuboidal cells containing large amounts of pigment in their apical portion. Tissues cultured in media containing 100 mg/l glutamine showed the same degree of differentiation as those in 350 mg/l but without the accompanying abnormalities (see §4 for details). Explants cultured in medium containing 0-1, 1 or 10 mg/l glutamine were also normal but were smaller in size as the concentration of glutamine was decreased.

C. Effect of glutamine-deficient medium

In contrast to eyes explanted in Waymouth’s medium containing any of the above concentrations of glutamine, those in glutamine-deficient Waymouth’s medium (zero glutamine concentration) were very small and showed slow, but normal, development (Plate 1, fig. F). Mitotic figures were observed less frequently in these explants and were never present deep within the nuclear layer of the optic cup. Occasionally pigment granules were observed in the outer layer of the cup but in smaller numbers than in medium with freshly added glutamine. The retinal rudiment remained relatively immature throughout the 5-day culture period and in most cases little further differentiation was observed.
Abbreviations: ae, Anterior epithelium of lens; $l$, lens; lf, lens fibers; ls, lens suture; nl, neural layer of retina; pl, pigmented layer of retina; $r$, retina (optic cup); rd, retinal diverticulum; se, surface ectoderm.

Fig. A. Day-11 embryonic eye after 6 days of culture in Waymouth’s medium showing inner and outer layers of the optic cup. $\times$ 177.

Fig. B. Day-11 embryonic eye after 6 days of culture in Waymouth’s medium showing well differentiated lens fibers. $\times$ 177.

Fig. C. Day-11 embryonic eye after 5 days of culture in Waymouth’s medium with freshly prepared glutamine (350 mg/l.). The lens is large and irregular in shape. The anterior epithelium is ill-defined and an abnormal lens suture is present. $\times$ 177.

Fig. D. Day-11 embryonic eye after 5 days of culture in Waymouth’s medium with freshly prepared glutamine (350 mg/l.) showing three individual masses of lens tissue ($l^1$, $l^2$, $l^3$) which have fused. $\times$ 177.

Fig. E. Day-11 embryonic eye after 5 days of culture in Waymouth’s medium with freshly prepared glutamine (350 mg/ml.) showing a retinal diverticulum. $\times$ 177.

Fig. F. Day-11 embryonic eye after 5 days of culture in Waymouth’s medium without glutamine. The lens shows normal differentiation although it has remained relatively small. The optic cup is immature. $\times$ 177.
Abbreviations: l, lens; lc, lens cavity; lp, lens placode; ls, lens suture; lv, lens vesicle; nl, neural layer of retina; oc, optic cup (future retina); pl, pigmented layer of retina; r, retina.

Fig. G. Day-11 embryonic eye after 1 day of culture in Waymouth's medium. The optic vesicle has invaginated to form the optic cup and a lens placode is visible. ×177.

Fig. H. Day-11 embryonic eye after 2 days of culture in Waymouth's medium, showing both inner and outer layers of the retina and the lens vesicle. ×177.

Fig. I. Day-11 embryonic eye after 3 days of culture in Waymouth's medium. The lens has separated from the surface ectoderm and its cavity is obliterated. The pigmented and neural layers of the retina are clearly defined. ×177.

Fig. J. Day-11 embryonic eye after 4 days of culture in Waymouth's medium showing formation of a lens suture. ×177.

Fig. K. Day-11 embryonic eye after 5 days of culture in Waymouth's medium. The ocular tissues as well as surrounding tissues appear healthy. ×177.

Fig. L. Day-11 embryonic eye after 5 days of culture in Waymouth's medium showing beginning degeneration in the ocular tissues. ×177.

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Beyond the optic cup stage. Lenses were also less developed, remaining small, regular in shape with well-defined primary lens fibers, and a clearly delineated anterior epithelium.

Thus, cultures in glutamine-deficient medium and in medium containing concentrations of glutamine up to and including 100 mg/l. exhibited varying degrees of normal differentiation, whereas cultures in medium containing 350 mg/l. differentiated abnormally in most cases.

4. Time-study of the cultures

In order to determine the rate of differentiation in culture, eye rudiments from 11-day-old embryos were fixed for histological examination on each day of the 6-day culture period. Since cultures in Waymouth’s medium containing 100 mg/l. glutamine exhibited the greatest degree of normal differentiation, they will be described in detail initially. In later paragraphs these cultures will be compared with those in medium containing 350 mg/l. glutamine as well as those in glutamine-deficient medium.

After 1 day of culture in medium with 100 mg/l. glutamine, the optic vesicle had begun to invaginate and the overlying surface ectoderm had thickened to form the lens placode (Plate 2, fig. G). This indicated that during the first 24 h of culture the rate of differentiation of the optic anlagen lagged about 12 h behind the in vivo rate. At this time the explants appeared to be in poor condition, giving the impression that they would completely degenerate in a short time. Many degenerating cells had been sloughed into the lumina of the brain vesicles. On the second day of culture the optic cup was usually well formed, consisting of an inner thick layer and an outer thin layer. The lens vesicle had developed but was still attached to the surface ectoderm (Plate 2, fig. H). Many degenerating cells were still observed in the explant. This period of culture (first 2 days) will be referred to hereafter as the ‘initial period of adaptation’. On the third day the explants seemed to have ‘recovered’, and had a different appearance than during the first 2 days of culture. Most of the remaining cells appeared healthy. Apparently, the necrotic cells had undergone complete cytolysis, since few could be seen. The lens vesicle now had separated from the surface ectoderm and in many cases its cavity was completely obliterated by primary lens fibers (Plate 2, fig. I). Mesenchymal cells sometimes intervened between the resealed surface ectoderm and the lens, forming a structure resembling the cornea. Occasionally, end-plates of the fibers of Müller could be observed forming the limiting membranes of the retina. Between the third and fourth days of culture, pigment granules appeared in the outer layer of the retina and on the fourth day the onset of suture formation was observed (Plate, fig. J). It is striking that although a vitreous body would have been forming at this stage in vivo, it was absent in eye cultures on the fourth day and in all subsequent stages examined.

The healthy appearance of the explants and their differentiation continued, and was at a peak (and necrosis at a minimum) by the fifth day of culture
(Plate 2, fig. K). Also, at this stage isolated areas of brain tissue were differentiating. Neural and/or glial nuclei were beginning to stratify into layers, and nerve fibers could be traced into the surrounding mesenchyme. By the sixth day explants often began to show degenerative changes (Plate 2, fig. L).

When explants cultured in the presence of 350 mg/l. glutamine were observed on each day of the culture period the eye rudiments followed the same sequence of differentiation described above for 100 mg/l. glutamine. However, less cellular degeneration was observed with 350 mg/l. glutamine during the first 2 days of culture and the 'initial period of adaptation' was therefore less pronounced. Eye rudiments in this group of cultures were larger (indicating a faster growth rate) and as late as the third day of culture were less well differentiated than those in 100 mg/l. glutamine. Between the third and fifth days, however, both growth and differentiation occurred rapidly, so that by the fifth day of culture the eyes were as well developed as those grown in Waymouth's medium with 100 mg/l. glutamine but had in addition the abnormalities previously described. The survival rate of the tissues beyond the fifth day of culture was not enhanced by increased concentrations of glutamine.

When examined on each day of the culture period eye explants in glutamine-deficient medium were observed to be smaller and to reach their maximum differentiation more rapidly than explants in the presence of all concentrations of freshly prepared glutamine. However, the degree of maturation achieved without glutamine was always less than that seen in its presence. For example, lenses in a glutamine-deficient medium were usually separated from the surface ectoderm by the second day of culture but suturing of lens fibers was never observed. In contrast, lenses in the presence of fresh glutamine could remain attached to the surface ectoderm through the third day of culture, but between the third and fifth days sutures might be observed.

**DISCUSSION**

These studies establish the fact that early embryonic rat eye rudiments (optic vesicle stage) can undergo differentiation in culture in a completely defined chemical medium (Waymouth's). The rate of differentiation in the lens and retina under these *in vitro* conditions was considerably slower than the *in vivo* rate.

The vitreous body was notably absent under all *in vitro* conditions. Muthukkaruppan (1965) obtained similar results in early intact mouse eye rudiments at the same stage of development using Eagle's basal medium supplemented with chick embryo extract and fetal bovine serum. The absence of a vitreous body in culture may be due to lack of vascularization, particularly the failure of formation of the hyaloid artery and the tunica vasculosa lentis. Experiments on the eyes of early chick embryos have indicated that the lens controls development of the vitreous body which in turn largely determines gross size of the eye (Coulombre, 1956; Coulombre & Coulombre, 1964). Other observations by
Coulombre & Coulombre (1957) also show that the accumulation of vitreous tissue is responsible in large part for the normal growth of the cornea. In the present study, failure of the corneal epithelium to maintain its integrity as well as failure of retinal expansion may well be due to absence of the vitreous body.

The stage of development at the time of explanation appears to be of utmost importance for the success of early eye cultures in our system. Day-10 eyes along with most other organ rudiments dedifferentiated when the entire embryos were cultured. However, New & Stein (1964) have reported successful plasma clot cultures of intact mouse embryos at this stage when the amnion is left intact and the O₂ tension is increased to 60 %. In the present study the fetal membranes were removed prior to culture and the lack of this protective barrier may have contributed to the failure of these cultures to continue differentiation. Wolff (1957) emphasized the importance of a limiting membrane for the survival of many types of tissues in organ cultures. The lack of an epithelial capsule may also have been responsible for the widespread degenerative changes observed in day-12 explants. This idea was further supported when it was noted that day-11 explants remained more viable if the intact epithelium of the crown of the head was in contact with the medium rather than the cut (non-epithelialized) surface. Further experiments would be required in order to determine whether day-10 and day-12 eye rudiments can differentiate in the culture system at hand.

Other factors studied which affected the culture system were the pH of the medium and the gas phase. Tissues showed a higher degree of survival when the pH of the medium was about 7.8; a neutral pH of 7.0 gave satisfactory results but was inferior to the slightly alkaline pH. The most effective gas phase was 95 % air/5 % CO₂ while 95 % O₂/5 % CO₂ caused the tissues to degenerate. Lucas & Trowell (1958) found 97 % O₂/3 % CO₂ beneficial for several types of organ cultures including intact postnatal rat eyes. However, in the case of the eye, the authors conceded that this high level of O₂ was probably tolerated because the tunica of the eye hindered diffusion of the gas into the innermost eye tissues.

Because the medium was entirely chemically defined, it was possible to use the organ culture system to test accurately the effect of a single component of the medium (glutamine) on the development of the eye rudiment. The eye cultures were very sensitive to changes in glutamine content. The fact that even 0.1 mg/l. of glutamine had an effect on differentiation showed the importance of a completely defined medium in such studies. The medium which yielded eye development with the greatest degree of normal differentiation contained between 0.1-100 mg/l. fresh glutamine. A concentration of 350 mg/l. of fresh glutamine in the Waymouth's medium resulted in a marked increase in growth rate as evidenced by the number of mitotic figures present and the size of the eye anlagen. Furthermore, this acceleration in growth rate resulted in a number of morphological aberrations of both the lens and the retina which were not observed when the medium used contained 0.1-100 mg/l. glutamine. Eyes cultured in Waymouth's medium deficient in glutamine were normal but even smaller than
those in Waymouth's medium with the lesser amounts of added glutamine. The importance of glutamine for cell culture growth was emphasized by Levintow & Eagle (1961). These findings may indicate that concentrations of glutamine which are necessary for the rapid growth of cell cultures may be deleterious for normal differentiation in organ culture where integrated growth of the various tissue components of the organ rudiment is essential. On the other hand, such a system may also be used to study factors involved in abnormal embryonic growth of various tissues and this aspect will be discussed in a subsequent paper.

SUMMARY

1. The eyes and surrounding tissues of 10-, 11- and 12-day-old rat embryos have been grown in organ culture in a chemically defined medium (Waymouth's) for periods up to 6 days.
2. Histological examination of these tissues revealed that differentiation was consistently superior in explants from day-11 embryos as compared with day-10 and day-12 tissues.
3. The lenses and retinas of day-11 embryos differentiated in culture, but at a rate somewhat slower than normally occurs in vivo.
4. The vitreous body was absent in all cultures and it is probable that this accounts for the failure of the retina and cornea to expand in proportion to the size of the lens.
5. The effects of varying culture conditions such as pH, gas phase, and position of the explant are described.
6. A remarkable sensitivity of day-11 eye tissues to a single component of the medium, glutamine, was observed and the effects of various concentrations of this component on eye differentiation were compared.
7. The time course of eye differentiation in the organ culture system is described as well as the differences observed with various levels of glutamine in the medium.
8. The possibility of using the system in studies on abnormal eye morphogenesis is suggested.

Résumé

Développement des yeux d'embryons de Rat, en culture d'organe.

I. Effet de la glutamine sur le développement normal et anormal, dans un milieu chimiquement défini.

1. Les yeux et les tissus avoisinants d'embryons de Rat de 10, 11, et 12 jours, sont cultivés pendant 6 jours, en culture d'organe, sur milieu chimiquement défini (le milieu de Waymouth).
2. L'examen histologique de ces tissus, montre que la différenciation est plus importante dans les explants d'embryons âgés de 11 jours que dans ceux d'embryons âgés de 10 et de 12 jours.
3. Le cristallin et la rétine d'embryons de 11 jours se différencient en culture, mais cependant, plus lentement qu'in vivo.
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4. Le corps vitré est absent, dans toutes les cultures, ceci est probablement dû au fait que la rétine et la cornée ne s'étendent pas en proportion des dimensions du cristallin.

5. Les effets de la variation de certaines conditions de culture, telles que le pH, la phase gazeuse, la position de l'explant sont étudiés.

6. On observe une remarquable sensibilité des tissus de l'œil de 11 jours à un seul constituant du milieu, la glutamine. Les effets de concentrations variées de ce constituant sur la différenciation de l'œil sont comparés.

7. La durée de la différenciation de l'œil en culture d'organe est étudiée, ainsi que les différences observées avec des taux variées de glutamine dans le milieu.

8. Il est suggéré d'utiliser la culture d'organes pour étudier la morphogénèse des yeux anormaux.

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