Expression of differentiation markers by chick embryo neuroretinal cells *in vivo* and in culture

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**SUMMARY**

Several markers of chick neuroretinal differentiation were monitored *in vivo* and in culture. All increase markedly between 7 and 20 days of embryonic development *in vivo*. *In vitro*, endogenous GABA levels decrease almost immediately, while other neuronal markers increase as *in vivo* for 2 to 5 days before declining (choline acetyltransferase, acetyl cholinesterase, glutamic acid decarboxylase). Neuronal cell surface markers (binding sites for tetanus toxin, α-bungarotoxin, muscimol), however, reach maximal levels only after 8 days *in vitro*. Glial markers such as carbonic anhydrase and hydrocortisone-induced glutamine synthetase activities are also expressed only transiently in culture.

**INTRODUCTION**

The differentiation of chick embryo neuroretinal (NR) cells has been studied extensively both *in vivo*, and *in vitro* using monolayer and aggregate culture techniques. In monolayer cultures of 6- to 8-day embryonic NR cells, several neuronal markers such as synaptogenesis (Crisanti-Combes, Privat, Pessac & Calothy, 1977; Vogel, Daniels & Nirenberg, 1976), choline acetyltransferase activity (CAT, EC 2.3.1.6.; Crisanti-Combes, Pessac & Calothy, 1978), and the number of α-bungarotoxin-binding sites (putative nicotinic acetylcholine receptors; Vogel *et al.* 1976; Betz, 1981) increase *in vitro* over a period of several days, in parallel with corresponding increases *in vivo* (see also Vogel & Nirenberg, 1976). This implies that retinal differentiation can proceed for some time *in vitro*, even under monolayer culture conditions where the tissue architecture has been disrupted.

The glial marker enzyme glutamine synthetase (GSase, EC 6.3.1.2.) does not normally appear in chick retinal Müller cells until the 16th day of development *in vivo*, but can be induced precociously by hydrocortisone (HC) and other corticosteroids from the 8th day onwards, both in retinal explants and in aggregate cultures of dissociated NR cells (Moscona, Mayerson, Linser & Moscona, 1980). However, GSase is apparently non-inducible in sparse

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monolayer cultures of these cells (Linser & Moscona, 1979), suggesting that the induction process is dependent on cell interactions reflecting some degree of histotypic tissue organization. The inducibility of this enzyme in aggregate cultures may be due to cell sorting and the partial re-establishment of normal tissue architecture within the ‘rosette’ structures formed by aggregation of dissociated embryonic chick NR cells \textit{in vitro} (Fujisawa, 1974).

A number of further anomalies in the differentiation of chick embryo NR cells in monolayer culture have been reported. Thus CAT activity is not sustained after its initial increase in such cultures, but rather falls sharply after 3 to 10 days \textit{in vitro}, depending on medium conditions (de Pomerai & Gali, 1981). GABA accumulation by retinal neuronal cells (de Pomerai & Carr, 1982) similarly declines steeply after about 7 days of culture (Guérinot & Pessac, 1979; de Pomerai, Carr, Soranson & Gali, 1982). Furthermore, the inhibitor sensitivity of the GABA uptake process changes during the first week of culture, from predominantly 2,4-diaminobutyric acid (DABA)-sensitive after 3 days (Hyndman & Adler, 1982) to predominantly DABA-insensitive after 6 to 8 days \textit{in vitro} (Hyndman & Adler, 1982; de Pomerai & Carr, 1982). In general, sharp decreases in the levels of particular neuronal markers considerably precede the physical loss of most morphologically recognizable neuronal cells from these cultures, which normally occurs between 15 and 25 days \textit{in vitro} (de Pomerai, Pritchard & Clayton, 1977; Okada, Itoh, Watanabe & Eguchi, 1975).

These \textit{in vitro} departures from the pattern of differentiation followed \textit{in vivo} are of particular interest in view of the ‘transdifferentiation’ processes which later supervene in long-term cultures of chick embryonic NR cells, leading to the appearance of well-differentiated lens (Okada \textit{et al.} 1975) and pigment (Itoh, Okada, Ide & Eguchi, 1975) cells after 20 to 30 days \textit{in vitro}. These ‘foreign’ cell types are apparently derived from the progeny of authentic NR cells (Okada, 1977; Okada, Yasuda, Araki & Eguchi, 1979), and their appearance can be blocked either by manipulating the culture conditions (de Pomerai & Gali, 1981, 1982) or by culturing freshly dissociated NR cells as aggregates (Okada, Nomura & Yasuda, 1983). The question therefore arises as to how these novel transdifferentiation pathways might be related to the loss of previously established patterns of normal retinal differentiation.

In this report we have studied a range of retinal differentiation markers characteristic of either neuronal or glial cell types. We find that these markers are lost in several phases during \textit{in vitro} culture, suggesting that some aspects of NR differentiation do not proceed at all in monolayer cultures, while others continue for between 2 and 5 days, and still others for about 10 days \textit{in vitro}. However, all of the markers studied decline to low levels by 20–30 days of culture, corresponding to the time at which pigment cell markers (tyrosinase and melanin; Itoh \textit{et al.} 1975) and lens crystallins (Okada \textit{et al.} 1975; de Pomerai \textit{et al.} 1977) begin to accumulate under the culture conditions used in these experiments. These
decreases may reflect actual cell loss, dedifferentiation, or overgrowth by undifferentiated proliferating cells.

MATERIALS AND METHODS

Fertile chicken eggs were obtained from Ross Poultry Ltd, Bilsthorpe, Notts. 

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\[ ^{14}\text{C}]\text{Acetyl CoA (59 mCi/mmole), } ^{1-^{14}\text{C}}\text{-DL-glutamic acid (52 mCi/mmole), } ^{3}\text{H}\text{muscimol (15.5 Ci/mmole) and } ^{3}\text{H}\text{propionylated } \alpha\text{-bungarotoxin (75 Ci/mmole) were all from the Radiochemical Centre (Amersham, Bucks.). Tetanus toxin and antitoxin were generous gifts from Dr R. O. Thomson (Wellcome Research Laboratories, Beckenham, Kent). Lyophilized FITC-conjugated goat anti-rabbit IgG was from Flow Ltd (Irvine, Scotland). Ovine brain glutamine synthetase, bovine erythrocyte carbonic anhydrase, Trypsin type I, glutamic acid decarboxylase and GABase were all from Sigma Ltd (Poole, Dorset). Culture-medium components and sera were from GIBCO-Europe Ltd (Paisley, Scotland), and tissue-culture disposables from Nunc Ltd.}

1. Cell Culture

Cultures of 9- or 10-day chick embryo neuroretinal (NR) cells were established as described previously (de Pomerai & Gali, 1981), at densities of about \(10^7\) cells per ml. Culture medium FH was as specified by de Pomerai & Gali (1982), i.e. Eagle’s MEM with Earle’s salts, 26 mm-NaHCO\(_3\), 2 mm-L-Glutamine, 100 i.u./ml penicillin, 100 \(\mu\)g/ml streptomycin, 5% horse serum, and 5% foetal calf serum from a single batch selected for its ability to support extensive transdifferentiation in NR cultures. All cultures were maintained at 37°C in a humid atmosphere of 5% CO\(_2\):95% air (v/v), the medium being changed every 2–3 days. Under these conditions, some 75% of inoculated cells become attached within 24 h, and net culture growth (representing proliferation of flattened glial-type cells) supervenes over net cell loss (mainly of neurone-like cells) after about 12 days in vitro (for details, see de Pomerai & Gali, 1982).

In the case of cultures used for GSase assays (Fig. 8), F medium containing 10% foetal calf serum and no horse serum was used (de Pomerai et al. 1982), either with or without 5 \(\mu\)M-hydrocortisone (HC) continuously present. This eliminated confusions introduced by traces of inducer (corticosteroid hormones) in the adult horse serum used for our standard FH medium. Explant cultures (Fig. 8 only) used fragments of NR tissue from embryos of different ages (5 to 19 days of incubation), which were maintained in F medium with or without HC for 48 h in bacteriological Petri dishes under continuous gyration (60 r.p.m.) on an orbital shaker.

2. Immunofluorescent staining with tetanus toxin

The procedure of Mirsky et al. (1978) was followed. Living NR cultures were washed thoroughly with at least six changes of phosphate-buffered saline (PBS)
between successive 60 min incubations in tetanus toxin (20 µg/ml), antitoxin (diluted 50-fold) and FITC-conjugated goat anti-rabbit IgG (reconstituted according to the manufacturers’ instructions and diluted 20-fold). After fixing for 30 min with 5% acetic acid: 95% ethanol (v/v), cultures were mounted in glycerol and immediately examined under a Leitz epifluorescence microscope with a 420 nm filter. Controls omitted the incubation in tetanus toxin.

3. Binding studies with radioactive ligands

Fresh retinal tissue (7 to 24 days of development) and batches of NR cultures were homogenized in distilled water and centrifuged at 25,000 g for 2 h at 4 °C. This procedure was then repeated to give a crude retinal membrane pellet.

i) For muscimol binding (based on the procedure of Sarlieve et al. 1980), pellets were homogenized in ice-cold 50 mM-Tris–citrate buffer (pH 7.1), the protein contents determined by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951), and each homogenate adjusted to a final concentration of 1 mg/ml. Five 100 µl aliquots were mixed with 100 µl of Tris–citrate buffer containing 0.2 µCi of [3H]muscimol (65 nM final) to determine total binding. Five further 100 µl aliquots were premixed with 4 µl of 100 mM-GABA (2 mM final) before addition of labelled muscimol as above, to estimate ‘non-specific’ binding not displaceable by excess GABA. These mixtures were incubated on ice for 60 min, centrifuged (14,000 g for 15 min at 4 °C) and the supernatants drawn off using a capillary aspirator device. Pellets were processed for counting as described previously for GABA-labelled culture material (de Pomerai & Carr, 1982). Mean differences between total and non-displaceable muscimol binding were taken to measure the GABA-displaceable binding for this ligand. In preliminary assays, four different concentrations of labelled muscimol were incubated with a crude membrane preparation from fresh 12-day embryonic retinal tissue, and the data subjected to Scatchard analysis. The 65 nM concentration found optimal for this material was used in all subsequent experiments.

ii) Binding studies using [3H]α-bungarotoxin were based on the methods of Shain et al. (1974) and of Carbonetto & Famborough (1979). The preparation of crude membrane homogenates of equal protein concentration (1 mg/ml final) was as above, except that the buffer used was 10 mM-Tris–HCl pH 7.8. Five 100 µl aliquots were mixed with 0.1 µCi [3H]bungarotoxin (8 nM final) in 60 µl of the same Tris buffer to estimate total binding; five similar aliquots were premixed with 8 µl of unlabelled 2 mg/ml α-bungarotoxin (Sigma; 2.5 µM final) prior to addition of labelled α-bungarotoxin as above, to estimate non-displaceable binding. After incubation for 60 min at 37 °C, samples were filtered through type EGWP 0.2 µm pore cellulose acetate filters (Millipore Ltd); the filters were then washed extensively with 10 mM-Tris buffer containing 0.1 % (w/v) bovine serum albumin (BSA), and dried for scintillation counting. Mean differences between the two sets of results were again taken to measure net displaceable binding of α-bungarotoxin. Preliminary experiments using different concentrations of
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Labelled ligand were performed for Scatchard analysis as above; again the optimal concentration for α-bungarotoxin binding with this material (8 nM) was used for all subsequent experiments.

4. Enzyme assay methods

All protein concentrations were determined as above by the Lowry method.

i) Choline acetyltransferase activity (CAT; E.C.2.3.1.6.)

Cultured or fresh retinal material was homogenized in PBS containing 0.4% Triton X-100 and 1 mM-EDTA, the homogenates centrifuged (5000 g for 10 min at 4 °C), and 10 μl aliquots of each supernatant assayed in triplicate as described previously (Crisanti-Combes, Pessac & Calothy, 1978; de Pomerai & Gali, 1981), using 0.01 mM final [14C]Acetyl CoA. Control assays (not shown) containing 100 μg/ml of the specific CAT inhibitor 1-(4-naphthyl vinyl)-pyridine (Calbiochem Ltd) gave at least 75% inhibition of CAT activity, suggesting that carnitine acetyltransferase activity is low in chick NR material (see also Crisanti-Combes et al. 1978).

ii) Acetylcholinesterase activity (AChE; E.C.3.1.1.7.)

AChE was assayed by the spectrophotometric method of Ellman, Courtney, Andres & Featherstone (1961), using acetylthiocholine iodide as substrate and dithiobisnitrobenzoic acid (DTNB) as the colorimetric reagent. The increase in absorbance at 412 nm was monitored at 5 min intervals over a period of 30–40 min, and the initial rate of reaction (usually between 5 and 15 min) used to calculate enzyme activity. At least three replicate assays were performed for each sample (means given in Fig. 6), and two independent sets of material were analysed. Control assays (not shown) using butyrylthiocholine iodide as substrate gave less than 10% of the activity measured with acetylthiocholine iodide, suggesting that true AChE activity predominates over non-specific cholinesterase activity in chick retinal tissue.

iii) GABA levels and Glutamic acid decarboxylase activity (GAD; E.C.4.1.1.15.)

The fluorimetric method of Gabellec, Recasens, Benezra & Mandel (1980) was used for determination of GABA concentrations in homogenates of fresh and cultured NR material. For all samples, triplicate GABA assays and a similar number of blanks (omitting α-ketoglutarate) were prepared, and the mean difference in fluorescence used to calculate the GABA concentration by reference to a set of standard assays containing 0–12 μM-GABA.

This method was also used to determine the amount of GABA formed by GAD activity in samples of retinal homogenates, as described by Gabellec et al. (1980), except that a pH of 7.0 was found to be optimal for chick NR material.
Results from parallel blank assays (omitting glutamic acid) were subtracted so as to determine the net amount of GABA formed by GAD activity.

A second set of GAD determinations was performed (in quadruplicate) according to the method of Guérinot & Pessac (1979), measuring the rate of evolution of $^{14}$CO$_2$ from 1-[$_{14}$C]DL-glutamic acid (0.5 μCi per assay).

Both assay techniques were standardized using known amounts of commercial GAD (Sigma), so that results obtained by the two methods could be directly compared in Fig. 4. In both cases, GAD activity was inhibited by >80% in the presence of 1 mm-aminooxyacetic acid (Guérinot & Pessac, 1979; results not shown).

iv) Glutamine synthetase activity (GSase; E.C.6.3.1.2.)

The colorimetric method of Moscona & Hubby (1963) was used with modifications as described by de Pomerai et al. (1982). Fresh retinal material (5 to 21 days of development), explant cultures (5- to 19-day NR tissue maintained for 48 h in F or F + 5 μM-HC media) and monolayer cultures of 9-day NR (maintained in these same two media for up to 24 days in vitro) were compared. Assays were incubated for 1 h at 37°C, together with tissue blanks omitting hydroxylamine; 0.6 M-Fe(NO$_3$)$_3$ in 40% trichloroacetic acid (Iqbal & Ottaway, 1980) was used as the colorimetric reagent for greater sensitivity. Standards containing 0 to 0.1 units of purified ovine brain GSase (Sigma) were assayed in parallel, and the final optimal densities read at 500 nm. Assays were performed at least in quadruplicate, and the net difference between experimentals and tissue blanks was used to calculate GSase activity in units per mg soluble protein.

v) Carbonic anhydrase activity (CA; E.C.4.2.1.1.)

CA activity was determined by the method of Kimelberg, Stieg & Mazurkiewicz (1982). Mean CA activity was determined from at least four replicate samples, using a reference series of standards containing known amounts of commercial CA (Sigma bovine erythrocyte CA, 2500 units/mg). CA activity was inhibited by more than 90% in the presence of 1 μM-acetazolamide (results not shown).

RESULTS

1) Fluorescent markers in NR cultures

Between 3 and 15 days of culture, tetanus toxin staining of retinal neurone-like cells, and particularly of their interconnecting neurite processes (Fig. 1A, B, D,
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Fig 1
is much more intense than the background staining observed when toxin is omitted (Fig. 1C, E, H). At later stages (20 days onwards), neurite processes mostly disappear and the remaining cells of neuronal morphology show little difference in staining intensity between the two procedures. At no stage is the sheet of flattened epitheloid cells (presumptive Müller glia) stained significantly by tetanus toxin, suggesting that these cells possess few if any binding sites for the toxin (see Mirsky et al. 1978). Tetanus-toxin-binding sites are thus present on many retinal neurone-like cells and processes, but not on the epitheloid (immature glial) cells during the first two weeks of culture in vitro (Fig. 1). Most cells and processes of neuronal morphology seem to stain positively in NR cultures from this embryonic stage, possibly because the photoreceptor cells (which do not stain with tetanus toxin in rat NR cultures; Beale, Nicholas, Neuhoff & Osborne, 1982) have not yet differentiated. Neurite processes appear to stain slightly more strongly after 8 or even 15 days as compared to 3 days of culture, suggesting that tetanus-toxin-binding sites may increase in number or become more accessible during in vitro culture.

2) Ligand binding by crude membrane preparations of NR in vivo and in vitro

Labelled α-bungarotoxin (αBTX) and muscimol have been used widely as 'specific' ligands for nicotinic acetylcholine receptors (Vogel & Nirenberg, 1976; Vogel, Daniels & Nirenberg, 1976; Betz, 1981) and for GABA receptors (Sarliève et al. 1980) respectively. However, the precise binding specificity of both ligands is open to some dispute (Carbonetto & Famborough, 1979; de Feudis, 1980) and for this reason we refer to αBTX-binding sites and muscimol-binding sites.

It will be noted from the inserts in Fig. 2 that Scatchard analysis of [3H]αBTX binding at different ligand concentrations (Fig. 2A insert) suggests a single binding component, whereas a similar analysis for [3H]muscimol binding (Fig. 2B insert) suggests at least two components with differing affinities for the ligand. This point was not further explored for the purposes of the present study.

Net αBTX binding by crude retinal membrane preparations increases to much the same extent in culture as in vivo up till the 7th or even 10th day in vitro (equivalent to 16–19 days in vivo), and thereafter declines slowly, reaching low levels by 30 days of culture (Fig. 2A). This decline parallels the physical loss of neuronal cells from these cultures, which occurs mainly between 15 and 25 days in vitro (Okada et al. 1975; de Pomerai et al. 1977). Overall, this pattern is similar to that reported by Betz (1981).

The pattern for [3H]muscimol binding by crude retinal membranes is somewhat similar (Fig. 2B), except for a pronounced initial lag during which binding declines slightly in culture (7 days for muscimol binding as compared to only 3 days for αBTX binding; compare Figs 2A and 2B). Maximal muscimol binding is again observed after 9 to 11 days in culture, although these levels are markedly lower than at the corresponding stages in vivo (19 to 21 days of
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**A** α BUNGAROTOXIN BINDING

![Graph A](image)

**B** MUSCIMOL BINDING

![Graph B](image)

Fig. 2. Ligand binding by crude membrane preparations of neuroretinal cells in vivo and in vitro. Binding assays on crude retinal membrane preparations with [3H]α-bungarotoxin (Part A) and [3H]muscimol (Part B) were performed as described in Methods. In all cases, the net difference between total binding and non-displaceable binding (in the presence of excess unlabelled α-bungarotoxin or GABA, respectively) was calculated from at least five assays of each type. For each time point, the variance of the total binding assays was significantly different from that of the non-displaceable binding assays (shown using Snedecor's F test). Inserts show Scatchard analysis of ligand binding assayed under the same conditions with a fresh 12-day embryonic NR membrane preparation and four different concentrations of ligand (α-bungarotoxin for A, muscimol for B). For both parts:- • •, ligand binding by crude membranes from fresh retinal material (6-day embryo to 3-day post-hatch, i.e. 24 days). ○ --- ○, ligand binding by crude membranes from cultured retinal material (9-day embryonic NR used for the cultures in part A, 10-day NR for the cultures in part B).

Overall, the in vitro development of membrane binding sites for both αBTX and muscimol follows a time course similar to that noted for tetanus toxin binding, with maximal levels being attained between the first and second weeks of culture.

3) **Endogenous GABA levels**

Fig. 3 shows that endogenous GABA levels increase markedly during NR differentiation in vivo, particularly after the 13th day of development. By
Fig. 3. GABA content of neuroretinal cells in vivo and in vitro. Endogenous GABA levels were determined in retinal homogenates using the fluorimetric method of Gabellec et al. (1980), as described in Methods. Each point shows the mean and standard error calculated from five independent sets of fresh retinal samples (5- to 20-day embryos) and from four sets of cultures (starting with 9-day embryonic NR cells). Time points for which only two sets of data are available are given as simple means without standard errors, and should be regarded as merely indicative. •—•, GABA levels in fresh retina; ▲—▲, GABA levels in cultured 9-day retinal cells.

contrast, in cultures of 9-day NR cells GABA levels decline steeply, with some loss detectable even after 2 days of culture, reaching insignificant levels by the 12th day in vitro. This may reflect actual loss of GABA-containing cells, a failure of differentiation (e.g. insufficient GABA synthesis by GAD, but see 4 below), dedifferentiation, and/or spontaneous release of GABA from these cells (see Hyndman & Adler, 1982) during the first few days of culture.

4) Neurotransmitter-related enzymes (GAD, CAT, AChE)

The levels of glutamic acid decarboxylase activity (GAD) increase over a period of about 5 days in vitro, to much the same extent as over the corresponding period in vivo (Fig. 4). Thereafter, GAD activity rises steeply in vivo (13–20 days of development), but declines progressively in culture, reaching background levels by the 12th day in vitro. A somewhat similar pattern is apparent
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Fig. 4. GAD activity of neuroretinal cells in vivo and in vitro. GAD activity was determined in retinal homogenates (i) by fluorimetric determination of the GABA formed (cf. Fig. 3; Gabellec et al. 1980), and (ii) by trapping of the $^{14}$CO$_2$ evolved from 1-$^{14}$C]DL-glutamic acid (Guérinot & Pessac, 1979), as described in Methods. Both methods were standardized using known amounts of commercial GAD (Sigma). Each point shows the mean and standard error derived from at least four replicate assays. Open symbols; data derived by $^{14}$CO$_2$ evolution (Guérinot & Pessac, 1979). Filled symbols; data derived by fluorimetry of GABA formed (Gabellec et al. 1980). ○/●—○/●, GAD activity in fresh retina (7-day embryo to 2 days posthatch, i.e. 23 days). △/▲—△/▲, GAD activity in cultures starting with 9-day embryonic NR. Retinal GAD activity was inhibited by >80% in the presence of 1mm-aminooxyacetic acid (data not shown).

both for choline acetyltransferase (CAT, Fig. 5) and for acetylcholinesterase (AChE, Fig. 6), the initial increase in enzyme activity in culture being sustained for only 3 and 2 days respectively, followed by a decline in low levels by about the 12th day. In the case of CAT, the initial increase is quite dramatic, since this enzyme rises earlier in NR development (7 to 14 days) than do either GAD or AChE (which increase most rapidly after about 12 days in vivo). The normal developmental pattern for these three enzymes is therefore maintained for 5 days or less in cultures of 9-day embryonic chick NR cells, in apparent contrast to the neuronal membrane markers discussed above (1 and 2). Again, the respective roles of cell loss and of dedifferentiation in the subsequent decline of these enzyme activities remain to be elucidated. However, the majority of neurite processes are not lost until 10–15 days of culture under these conditions (Gali, unpublished observations; see also Fig. 1), while aggregates of morphologically recognizable neuronal cells remain abundant until at least 18 days in vitro.
5) Glial markers (GSase and CA)

Since glutamine synthetase (GSase) does not normally appear in the chick retina until 16 days of development in vivo (Moscona et al. 1980), its activity can only be studied in cultures of 9-day embryonic NR cells after precocious induction by hydrocortisone (HC; de Pomerai et al. 1982) Fig. 7A shows the appearance of GSase activity in fresh retina and in explants cultured in vitro for 48 h in standard medium with or without hydrocortisone present. It will be noted that detectable levels of GSase activity (higher than in fresh retina of corresponding total age) appear in explants cultured in the absence of HC, perhaps due to the presence of trace amounts of inducing agent in the unsupplemented medium (Ham’s F-12 medium shows this effect to an even more pronounced degree; Gali & de Pomerai, submitted). However, much higher levels of GSase activity are induced precociously by 5 μM-HC in explants of 8- to 15-day embryonic neural retina tissue, although the levels attained in vivo by the time of hatching (20–21 days) are not approached under these explant culture conditions (see also de

![Graph showing CAT activity of neuroretinal cells in vivo and in vitro. CAT activity was determined in homogenates of fresh and cultured retina by the method of Crisanti-Combes et al. (1978), modified as described in Methods. Each point gives the mean and standard error derived from at least six assays using two independent sets of fresh retinal material and two batches of cultures from 9-day embryonic NR. Retinal CAT activity was inhibited by at least 75% in the presence of 100 μg/ml 1-(4-naphthylvinyl) pyridine (specific CAT inhibitor; Crisanti-Combes et al. 1978), suggesting that carnitine acetyltransferase activity is minimal in chick retinal tissue. - - - - , CAT activity in fresh retina (5 to 19 days of embryonic development). ▼ - - - ▼, CAT activity in retinal cultures (from 9-day embryonic NR).]
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Pomerai et al. 1982). In most respects these findings confirm those of Moscona and co-workers (reviewed in Moscona et al. 1980).

Fig. 7B shows that 9-day embryonic NR cultures maintained in the absence of HC develop low levels of GSase activity (maximal after 5 days in vitro), comparable to those obtained with retinal explants in the same medium (Fig. 7A). In the continuous presence of 5 μM-HC, however, much higher levels of GSase are induced, reaching a maximum (6-fold higher than in controls) after 8 days in vitro, but thereafter declining back to control levels by 20 days despite the continued presence of inducer. One possible reason for this decline is the onset of rapid proliferation among the epithelial cells, especially after about 12 days.

Fig. 6. Acetylcholinesterase activity in neuroretinal cells in vivo and in vitro. Acetylcholinesterase activity was assayed in retinal homogenates by the method of Ellmann et al. (1962), as described in Methods. Each point gives the mean activity derived from three determinations of initial reaction rate. Two independent sets of fresh retinal material and two batches of 9-day embryonic NR cultures were used, the data from each set being distinguished by open (set I and batch I) and closed (set II and batch II) symbols respectively. ○/●—○/, AChE activity in fresh retina (6-day embryo to 3 days posthatch, i.e. 24 days). △/▲—△/, AChE activity in cultures of 9-day embryonic NR cells. Samples showing significant cholinesterase activity were also assayed with butyrylthiocholine iodide as substrate. These gave less than 10% of the activity measured using acetylthiocholine iodide as substrate, suggesting that AChE activity greatly predominates over non-specific cholinesterase activity in chick retinal material (data not shown).
A IN VIVO AND EXPLANTS

Part A shows the levels of GSase activity assayed (as described in Methods) in fresh and explanted retina (the latter maintained for 48 h in culture medium at 37°C prior to assay). (i) •–•, fresh retina immediately after excision. (ii) ———, fresh retina explanted into culture medium without hydrocortisone for 48 h. (iii) ○——○, fresh retina explanted into culture medium containing 5 μM-hydrocortisone for 48 h. Note that in the case of (ii) and (iii), the number of days is taken as including the 48 h explant culture period (i.e. 9-day points represent explants of 7-day NR, etc).

Part B similarly shows the levels of GSase activity determined in cultures of 9-day embryonic retina maintained in culture medium either with or without 5 μM-hydrocortisone continuously present. •——•, retinal cultures maintained in the absence of hydrocortisone. ○——○, retinal cultures maintained in the continuous presence of 5 μM-hydrocortisone. Each point gives the mean and standard error derived from 4–6 replicate assays, after prior subtraction of appropriate tissue blanks. The assay reaction was standardized on each occasion by inclusion of a reference series containing 0 to 0.1 units of purified ovine brain GSase (Sigma).
of culture. The maximum level of induced GSase activity in these cultures is comparable to that obtained with explants of 8- or 12-day NR after a 48 h exposure to HC (10- and 14-day points in Fig. 7A).

Carbonic anhydrase (CA) activity decreases in vivo between 7 and 15 days of development, but then rises steeply towards the time of hatching (Fig. 8A). A similar pattern was reported by Linser & Moscona (1981), who also showed by immunohistochemistry that the CA-C enzyme becomes progressively localized in Müller glia during embryonic development of the chick. Thus the initial decline in CA activity in vivo may reflect loss of the enzyme from retinal neuronal cells, followed by an increase in activity confined to the Müller cells during their maturation. In culture, CA activity is mostly lost during the first few days in vitro (Fig. 8B), perhaps for similar reasons, but thereafter increases only slightly before plateauing and eventually declining. This might suggest a failure

![Graph](image)

Fig. 8. Carbonic anhydrase activity of neuroretinal cells in vivo and in vitro. All data shown in this figure were derived by the method of Kimelberg et al. (1982), as described in Methods. Each point gives the mean and standard error derived from at least four replicate assays on retinal homogenates from two sets of embryos and three sets of cultures. Part A: ▲—▲, CA activity in fresh retina (9- to 20-day embryos). Part B: ●—●, CA activity in retinal cultures (from 9-day embryonic NR). The unit definition of Kimelberg et al. (1982) was verified by use of commercial carbonic anhydrase (2500 units/mg) from bovine erythrocytes (Sigma). 1 μM-acetazolamide inhibited CA activity by >90% (data not shown).
of differentiation among the epitheloid cells (immature glia?) in vitro, again possibly as a consequence of active cell proliferation.

DISCUSSION AND CONCLUSIONS

Points relating to particular marker systems have mostly been dealt with above, hence this section will discuss the general trends and their possible significance. All of the neuroretinal markers studied here are declining or have already fallen to low levels by about 20 days in culture, as compared to steep increases in vivo during later embryonic development.

A Glial markers

The two glial markers (GSase and CA) are of particular interest since putative precursors of the cells in which they are localized (Müller glia) are not lost from NR cultures in vitro, but rather proliferate actively during the second and third weeks of culture (see Okada et al. 1975). Carbonic anhydrase (CA) activity fails to increase in NR cultures at the time when it becomes glial-specific and rises sharply in vivo (Fig. 8; Linser & Moscona, 1981). Glutamine synthetase activity (GSase) can be induced precociously by hydrocortisone in dense cultures of embryonic NR cells containing numerous neuronal aggregates. However, the induced levels of activity are not maintained even in the continued presence of inducer (HC), suggesting some degree of dedifferentiation among the retinal glial cells in vitro. This may be related to the disappearance of neurite processes and eventual loss of overlying neuronal aggregates between about 10 and 25 days in vitro, and/or to the rapid proliferation of epithelial cells leading to an increase in total cell numbers after about 12 days of culture (de Pomerai & Gali, 1982). In the latter case, individual 'glial' cells (expressing GSase) may dedifferentiate prior to proliferation, or a population of more differentiated glial cells (possibly those located underneath neuronal aggregates?) may simply become diluted due to overgrowth of rapidly dividing undifferentiated epithelial cells not expressing GSase. It is of interest to note that transdifferentiation into lens cells is both delayed (by some 10 days) and reduced in extent (by five- to six-fold) in the continuous presence of HC (de Pomerai et al. 1982). This might suggest that retinal glial cells in vitro are faced with a choice between normal and 'foreign' differentiation pathways (cf. de Pomerai & Gali, 1981), the latter being partially inhibited and the former at least temporarily promoted in the presence of HC (de Pomerai et al. 1982). There are indications that HC may also inhibit glial proliferation, especially in cultures of younger embryonic NR cells (de Pomerai et al. 1982), and that neuronal cell survival is improved in its presence.

B Neuronal markers

All other markers studied here are confined to NR neuronal cells of various types, none being detectably present in cultures from which neuronal cells have
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been removed (cf. de Pomerai & Carr, 1982). When these markers are monitored in vitro, three broad categories may be distinguished.

(i) Markers which decline from the beginning of the culture period. The only clear example in this category is the endogenous GABA content of NR cells, which is already slightly lower after 2 days in vitro as compared with the starting material (9-day embryonic NR).

(ii) Markers which show a pronounced increase during the first few days of culture in vitro, paralleling that which occurs over the corresponding period in vivo, but which later decline markedly reaching low levels by about 12 days of culture. Into this category fall the neurotransmitter-synthesizing enzymes GAD and CAT, and also the degradative enzyme AChE (studies on GABA-transaminase are currently in progress). Previous investigations of GABA accumulation by cultures of chick embryo NR cells (Guérinot & Pessac, 1979; de Pomerai et al. 1982) also suggest a maximum between 3 and 7 days in vitro, followed by a steep decline to low levels by 12 days, although corresponding in vivo data is not available in these studies.

(iii) Markers which reach maximum levels after 8 to 10 days in vitro. These include the binding sites for α-bungarotoxin, muscimol and probably tetanus toxin. It will be noted that all three are present on neuronal cell surface membranes. Moreover, all three markers decline in parallel with the physical loss of neuronal cells from these cultures (mainly between 15 and 25 days in vitro). This group may represent merely an extension of category (ii) above, although the presence of abundant cell surface ligand-binding sites at a stage of culture (10 days in vitro) when internal enzyme markers characteristic of neurones have fallen to low levels, suggests a difference that may repay further investigation.

POSSIBLE MECHANISMS

Three basic explanations may be advanced to account for the eventual loss of these markers of normal retinal differentiation during prolonged culture: (a) dissociation and loss of the cells expressing these markers; (b) dedifferentiation of such cells; (c) overgrowth by undifferentiated cells. All three probably play some role in chick NR cultures; e.g. (a) most probably accounts for the loss of neuronal cell surface markers (category B iii, above), while (c) may be involved in the loss of glial markers (A above). However, (c) is unlikely to become important until active culture growth (epithelial cell proliferation) supervenes over net cell loss (mainly of neurones) during the second and third weeks of culture, although some cell proliferation probably begins at an earlier stage than this. The maintenance or increase of some neuronal markers (cell surface ligand-binding sites) but not others (e.g., neurotransmitter-related enzyme activities) between the 5th and 10th days of culture also argues against explanation (c), since differentiated neuronal cells of any type are unlikely to divide in these cultures.
Since most of the markers studied here are probably localized in different (or at best overlapping) subpopulations of neurones, it is not possible to establish whether two or more markers present in the same cell type disappear simultaneously or at different rates prior to actual cell loss. The former would merely indicate differential survival of the various cell types under these culture conditions, while the latter might suggest progressive dedifferentiation. We hope to investigate this question using purified populations of particular retinal neuronal types, recombined if necessary with neurone-free epithelial monolayers (de Pomerai & Carr, 1982). One candidate for such studies would be the nicotinic cholinceptive cells identified by their ability to bind FITC-conjugated α-bungarotoxin (de Pomerai, Takagi, Kondoh & Okada, submitted). Unfortunately the putative neurotransmitters synthesized by these cells (mainly ganglion cells; Morgan & Mundy, 1982) have not yet been identified in chick.

In many respects, the sequence of marker loss in NR cultures resembles that observed in neurones deprived of their presynaptic input, e.g. following nerve section (see review in Duce & Keen, 1980). Thus endogenous neurotransmitter levels decline first, followed later by decreases in the activities of transmitter-synthesizing and -degrading enzymes, and finally (following a transient increase) by a decline in the number of putative neurotransmitter receptor sites. This analogy raises the interesting possibility that retinal differentiation in situ may depend crucially upon the pattern of synaptic contacts which become established between different classes of retinal neurone (and also non-synaptic contacts with Müller glia) during embryonic development. This pattern will be completely disrupted during tissue dissociation, and is unlikely to be re-established under 'monolayer' culture conditions. However, some degree of histotypic layering (indicative of cell rearrangement and possibly formation of normal synaptic contacts) can be achieved in aggregate cultures of dissociated chick embryo NR cells (Fujisawa, 1974). This may explain why such aggregate cultures (i) maintain normal retinal differentiation markers and (ii) fail to transdifferentiate even after prolonged in vitro culture (Okada et al. 1983), contrasting sharply on both counts with 'monolayer' cultures of the same cells. Further studies involving aggregate cultures are currently in progress.

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