

# DEVELOPMENTAL DECLINE IN DNA REPAIR IN NEURAL RETINA CELLS OF CHICK EMBRYOS

## Persistent Deficiency of Repair Competence in a Cell Line Derived from Late Embryos

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### ABSTRACT

Neural retinas of 6-day-old chick embryos synthesize DNA and are able to carry out DNA excision repair. However, in contrast to the situation in human cells, the maximum rate of repair induced by *N*-acetoxy acetylaminofluorene (AAAF) is no greater than that induced by methyl methanesulfonate (MMS). With advancing differentiation of the retina in the embryo, cell multiplication and DNA synthesis decline and cease, and concurrently the cells lose the ability to carry out DNA excision repair. Thus, in 15–16-day embryos, in which the level of DNA synthesis is very low, DNA repair is barely detectable. If retinas from 14-day embryos are dissociated with trypsin and the cell suspension is plated in growth-promoting medium, DNA synthesis is reinitiated; however, in these cultures there is no detectable repair of MMS-induced damage, and only low levels of repair are observed after treatment with AAAF. A cell line was produced, by repeated passaging of these cultures, in which the cell population reached a steady state of DNA replication. However, the cell population remained deficient in the ability to repair MMS-induced damage. This cell line most likely predominantly comprises cells of retino-glial origin. Possible correlations between deficiency in DNA repair mechanisms in replicating cells and carcinogenesis in neural tissues are discussed.

Similar reactions are involved in DNA replication and DNA excision repair; this raises questions concerning the ability of nonreplicating cells to deal with damage to the genome. There is evidence that in some systems a correlation exists between the ability of cells to replicate DNA and their ability to carry out excision repair. (a) The differentiation of the myocyte to the postmitotic state is accompanied by a decrease in unscheduled DNA synthesis induced by ultraviolet (UV) light (28), by alkylation with methyl methanesulfonate

(MMS), (9) or by treatment with 4-nitroquinoline-1-oxide (4).<sup>1</sup> (b) The amount of nonsemiconservative DNA synthesis induced in populations of rodent cells by treatment with MMS in vitro is corre-

<sup>1</sup> Note added in proof: Peleg, Raz, and Ben-Ishai (1976, *Exp. Cell Res.* **104**:301–376) have recently shown that successive transfer of embryonic mouse cells in vitro is accompanied by a loss of excision repair capability for UV-induced damage along with a pronounced reduction in growth rate.

lated with the proportion of dividing cells in the population (13). (c) Human peripheral blood lymphocytes have low excision repair activity, and this activity is greatly increased when DNA synthesis is induced by mitogen treatment (26). (d) Cultures of murine neuroblastoma cells differentiate to a postmitotic state upon removal of serum from the medium (27), and such postmitotic cells are defective in the rejoining of single strand breaks introduced by gamma ray irradiation (3). This deficiency may be a characteristic of the postmitotic (as opposed to the  $G_0$ ) state (14).

In the neural retina of the chick embryo, cells proliferate during the first 12 days of embryonic life. Thereafter, the rate of cell division declines rapidly and eventually DNA synthesis practically ceases (19). This transition from a dividing to a nondividing cell population, and the ease with which relatively large numbers of neural retina cells can be obtained from chick embryos at different stages of development, make this an attractive tissue in which to study the relationship between DNA replication and DNA repair in embryonically developing cell populations. Furthermore, cells from retinas in which DNA synthesis has ceased can be stimulated to reinitiate DNA replication by dissociating the retina with trypsin and culturing the resultant cells *in vitro* with or without treatment with lectins (12). This makes it possible to examine experimentally both the loss of DNA repair capacity during differentiation and the effect of a reinitiation of replicative capacity on the ability to carry out excision repair.

## MATERIALS AND METHODS

### Cell Preparation

**SHORT-TERM CULTURES FOR DNA REPAIR STUDIES:** Neural retina tissue was dissected aseptically from eyes of 6-, 7-, 8-, 10-, 12-, 14-, 15- or 16-day chick embryos. Suspensions of single cells were prepared by incubation in 0.3% solution of pure trypsin (Armour and Co., Ltd., Eastbourne, Eng.) in  $Ca^{++}$ - and  $Mg^{++}$ -free Tyrode's solution (CMF) for 20 min at 37°C under a 5%  $CO_2$ -air mixture. After thorough washing in CMF, the tissue was mechanically dispersed by pipetting in Medium 199. Approximately  $10^7$  cells were dispensed into 25-ml plastic tissue culture flasks (BBL & Falcon Products, Becton Dickinson & Co., Cockeysville, Md.) containing 5 ml of Medium 199 (Grand Island Biological Company, Grand Island, N. Y.) supplemented with 10% fetal calf serum (FCS) (Grand Island Biological), penicillin and streptomycin. After gassing with 5%  $CO_2$ -air mixture, the flasks were incubated at 37°C on a gyratory shaker (12).

**LONG-TERM CELL CULTURES:** Cell suspensions were prepared from retinas of 14-day embryos by trypsinization of the tissue; approximately  $2.10^7$  cells were seeded into 25  $cm^2$  Falcon tissue culture flasks containing 5 ml of Medium 199. After 2-h incubation at 37°C to allow attachment, the medium was replaced with 5 ml of Medium 199 containing 10% FCS. The attached cells were incubated at 37°C in a humidified  $CO_2$  incubator. The medium was renewed every fourth day.

**ESTABLISHMENT OF CELL LINE:** Ten-day-old cultures that were originally started with retina cells from 14-day embryos were subdivided approximately 1:5 by brief trypsinization (2–5 min, 0.25% trypsin) into large (75  $cm^2$ ) Falcon tissue culture flasks containing 10 ml of Medium 199 + 10% FCS. This procedure (trypsinization and 1:5 dilution) was repeated after about 2 wk. Repair experiments were carried out 8–10 days after subdivision of cultures. The culture medium was changed every fourth day.

### Measurement of DNA Repair Synthesis

Methyl methanesulfonate (MMS) obtained from Eastman Organic Chemicals (Div., Eastman Kodak Co., Rochester, N. Y.), was redistilled under vacuum before use. Fresh stock solutions were prepared in phosphate-buffered saline for each experiment.

*N*-acetoxy acetylaminofluorene (AAAF), obtained from Dr. J. A. Miller (University of Wisconsin, Madison, Wis.), was dissolved in sterile dimethyl sulfoxide (DMSO) before use.

The standard protocol for the estimation of DNA excision repair was: 30-min preincubation with 10  $\mu M$  of the inhibitor cytosine arabinoside (Ara C) (Calbiochem, San Diego, Calif.), followed by the simultaneous addition of drug and 10  $\mu Ci ml^{-1}$  [ $^3H$ ]thymidine ([ $^3H$ ]TdR) (13–17 Ci/mmol) (Schwarz/Mann Div., Becton Dickinson & Co., Orangeburg, N. Y.). Repair was allowed to continue for 60 min, after which the cells were washed twice in 10 ml of phosphate-buffered saline (PBS) by centrifugation and resuspension (for cell suspensions) or by aspiration (for attached cells).

In experiments in which cell suspensions were used, the cells were resuspended in 1 ml of saline citrate (SSC) and lysed by addition of sodium dodecyl sulphate (SDS) to a final concentration of 0.2%. The lysates were taken through 3 cycles of freezing and thawing (dry ice/ethanol – 37°C) and digested with RNase and pronase. Before application to benzoyleated naphthoyleated *o*-(diethylaminoethyl)cellulose (BND-cellulose), the lysates were sheared by passage 5 times through a 20 gauge needle.

The procedure for cells in monolayer cultures was similar, except that cells were lysed *in situ* in 2 ml (25  $cm^2$  flasks) or 5 ml of SSC (75  $cm^2$  flasks). The lysates were then treated in the flasks as described above.

The methodology has been described in detail elsewhere (25). Cell lysates diluted in 0.3 M buffer (0.3 M NaCl, 0.1 mM EDTA, 10 mM Tris-HCl, pH 7.5) were

applied to BND-cellulose columns (0.75 g of resin) in 5-ml plastic syringes. The columns were step-eluted with 10 ml of 0.3 M buffer, 10 ml of 1.0 M buffer (1.0 M NaCl, 0.1 mM EDTA, 10 mM Tris-HCl, pH 7.5) and 10 ml of 50% formamide in 1.0 M buffer. The amount of native DNA in the 1.0 M buffer eluate was calculated from the absorbance at 260 nm, and the specific activity of repair, cpm/ $\mu$ g DNA, was calculated after precipitation of the DNA and determination of radioactivity as previously described (5).

### DNA Polymerase Assay (6)

10 retinas (about  $10^9$  cells) were pooled, suspended in 1 ml of ice-cold buffer A (10 mM Tris-HCl, 5 mM 2-mercaptoethanol (MSH) pH 7.5) and homogenized by five gentle strokes in a Dounce homogenizer. The resulting homogenate was centrifuged (2,000 rpm, 10 min, 0°C) in a Sorvall refrigerated centrifuge (DuPont Instruments, Sorvall Operations, Newtown, Conn.), and the supernate was kept on ice. Unbroken nuclei were extracted with 0.5 ml of buffer B (0.2 M potassium phosphate, 5 mM MSH, pH 7.5) for 30 min at 0°C. After a second low-speed centrifugation, the supernates were combined and centrifuged at 38,000 rpm for 90 min in the Type 40 rotor of the Beckman L3 ultracentrifuge (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.). The resulting supernate was stored frozen at -60°C in small aliquots.

The standard assay mixture (200  $\mu$ l) contained: KCl (2.5 mM),  $MgCl_2$  (8 mM), dATP, dCTP, dGTP (0.2 mM each), [ $^3H$ ]dTTP (0.2 mM: 61 cpm/pmol), 2-MSH (5 mM), Tris-HCl, pH 7.5, at 37°C (50 mM), bovine serum albumin (50  $\mu$ g/ml), 9  $\mu$ g of "activated" salmon sperm DNA (15), and extract (10-50  $\mu$ l).

DNA polymerase  $\beta$  was assayed under identical conditions, except that 2-mercaptoethanol was replaced with *N*-ethyl maleimide (NEM) (1 mM).

After 30 min at 37°C the reaction was stopped and the DNA was precipitated by the addition of 1 ml of 5% TCA and 100  $\mu$ l of bovine serum albumin (1 mg/ml). The precipitated material was collected by suction onto glass-fiber disks (Whatman GF/C, Whatman Inc., Clifton, N. J.), washed sequentially with 5% TCA, water and ethanol, dried and counted.

Activity was calculated as picomoles dTTP incorporated per hour per milligram protein in the range over which incorporation was linear with extract concentration.

### Assay for Thymidine Kinase

Assay mixtures (100  $\mu$ l) contained  $MgCl_2$  (2.5 mM), 100 mM Tris-HCl, pH 7.8, ATP (5 mM), bovine serum albumin (1 mg/ml), dithiothreitol (2 mM), unlabeled thymidine (50  $\mu$ M), [ $^3H$ ]thymidine (10-17 Ci/mmol, 10  $\mu$ Ci/ml), and 1-10  $\mu$ l of extract. It was not necessary to further purify the [ $^3H$ ]thymidine since the proportion of contaminating phosphorylated products was less than

0.06% as determined from the radioactivity of a 10- $\mu$ l aliquot remaining absorbed to DEAE paper after washing in Tris-HCl, pH 7.8, following incubation in the absence of cell extract. Tubes were incubated at 37°C for 20 min, after which the reaction was terminated by heating for 3 min at 90°C and cooling in ice. Phosphorylated products were determined by adsorption to DEAE paper (Whatman DE 81). Replicate 10- $\mu$ l aliquots from each tube were spotted onto 1-cm squares of DEAE paper and dried in a stream of warm air. The paper strips were then washed in four separate batches (500 ml) of 4 mM Tris-HCl, pH 7.8 (10 min each wash), dried, and each square was placed in a scintillation vial. To each vial was added 0.5 ml of 2.0 M NaCl and 10 ml of Instagel (Packard Instrument Co., Downers Grove, Ill.). The total available radioactive thymidine was determined from a 10- $\mu$ l aliquot spotted onto paper which was not washed with Tris-HCl. The activity was calculated as picomoles TdR phosphorylated per 10 min per microgram protein in the range over which the assay was linear with increasing amounts of extract.

Protein determinations were made by the method of Lowry et al. (17). DNA concentrations were estimated from absorbance at 260 nm after adsorption of cell lysates to BND-cellulose columns and elution of the DNA with 1.0 M buffer.

### Radioactive Compounds

[9- $^{14}C$ ]AAAF (21 mCi/mmol) was purchased from ICN and dissolved in sterile DMSO before use. [ $^{14}C$ ]MMS (52 mCi/mmol) was obtained from Amersham-Searle Corporation. After removal of the ether by evaporation, the radioactive MMS was dissolved in phosphate-buffered saline at a concentration of 1 mg/ml.

## RESULTS

### DNA Repair in Primary Cultures of Retinal Cells

ESTABLISHMENT OF A PROTOCOL FOR THE MEASUREMENT OF DNA REPAIR: DNA repair synthesis can be separated from replicative synthesis by using columns of BND-cellulose. This material retains DNA with single-stranded regions while native DNA passes through the column on washing with 1.0 M NaCl. In the presence of an inhibitor of DNA chain elongation, all radioactive thymidine incorporated by replicative synthesis will be retained by the column, whereas thymidine incorporated by repair synthesis will be found throughout the DNA and will be eluted with NaCl. The separation of replicative from repair DNA synthesis depends upon nearly complete inhibition of semiconservative synthesis, since extensive chain elongation will result in the

presence of radioactive thymidine in regions sufficiently distant from the growing point to appear in the NaCl eluate from BND-cellulose. With both human lymphoma cells and lymphocytes, about 98% inhibition of replicative synthesis can be obtained with 10 mM hydroxyurea. However, retina cells from 8-day embryos were found to be somewhat resistant to inhibition by hydroxyurea (Table I). This resistance resulted in a high specific radioactivity (cpm/ $\mu$ g) of the DNA from untreated cells in the 1 M BND-cellulose eluate, owing to the "leakage" of products of replicative synthesis into this fraction. Subsequent experiments were therefore carried out in the presence of 10  $\mu$ M cytosine arabinoside (AraC) which produced the required inhibition of replicative synthesis without disturbing repair synthesis, as measured in the 1.0 M NaCl eluate (Table I). There was good agreement between the observed levels of repair (cpm/ $\mu$ g DNA) in the presence of either inhibitor, indicating that AraC did not inhibit excision repair in these cells.

**THE EFFECT OF EMBRYONIC AGE ON LEVELS OF DNA REPAIR:** Cell suspensions obtained by trypsin-dissociation of retinas from embryos of different ages were treated with a range of concentrations of the two DNA repair-inducing agents, MMS and AAAF (Fig. 1). MMS was chosen as a representative of agents which induce "X-ray like" repair in human cells with repair patches of only 3-4 nucleotides (21, 24). AAAF produces "UV-like" repair in human cells with patch sizes of the order of 100 nucleotides (24). As observed with human cells, there was a dose-dependent increase in the specific radioactivity of the DNA eluted with 1.0 M NaCl from BND-cellulose, followed by a decline at the higher concentrations of both drugs. There are two main points of interest.

(a) Although the overall shapes of the dose-response curves are qualitatively similar for both agents, the level of repair synthesis at any particular concentration of either agent decreases with the embryonic age of the cells (Fig. 2*b*). The decrease in ability to carry out excision repair occurs over that period of embryonic development during which the retina cell population is differentiating to a nondividing state (Fig. 2*a*).

(b) The maximal rate of repair obtained at the different ages is not significantly different for the two compounds. The mean ratio of the maximal specific repair activities (AAAF max/MMS max) observed for the different ages is  $1.2 \pm 0.16$ . This

TABLE I  
*Comparative Specific Activities of Repair in the Presence of Hydroxyurea (HU) and Cytosine Arabinoside (AraC)*

AAAF concentration	Specific activity in presence of				"Repair specific activity" (mean treated - mean control)	
	HU		AraC		HU	AraC
	$\mu$ g/ml	cpm per $\mu$ g DNA	cpm per $\mu$ g DNA	cpm per $\mu$ g DNA	cpm per $\mu$ g DNA	cpm per $\mu$ g DNA
Control	0	75.4	8.6, 3.3	0	0	0
	10	81	17.5	5.6	11.6	11.6
	20	109	35.8, 45.4	33.8	34.7	34.7
	50	113.6	36.1, 43.1	38.2	33.7	33.7

Inhibition by HU 92.6%, inhibition by AraC 98.9%. Cell suspensions derived from trypsinization of 8-day chick retinas were incubated in the presence of either hydroxyurea (10 mM) or cytosine arabinoside (10  $\mu$ M) for 30 min at 37°C in Medium 199 containing 10% fetal calf serum. AAAF in DMSO was then added to the final concentrations shown, along with [<sup>3</sup>H]thymidine (10  $\mu$ Ci/ml, 11-17 Ci/mmol), and repair was allowed to proceed for a further 60 min at 37°C. Cells were harvested, washed, and lysates were prepared for chromatography on BND-cellulose as described in Materials and Methods. The inhibition of DNA synthesis was calculated from a separate culture which received only [<sup>3</sup>H]thymidine for 60 min. The specific activity of repair was determined from absorbancy (260 nm) and radioactivity of the material eluting with 1.0 M NaCl buffer. Replicate determinations of repair activity were carried out within the same experiment where indicated, and the repair specific activity was calculated by subtraction of the control specific activity from the treated specific activity (or mean where appropriate).

is to be compared with a ratio of about 5 obtained with a human cell line.

These data were obtained over the range of concentrations of MMS and AAAF that had been used to study DNA repair with other cell lines. The drug concentrations which induced maximal repair in the present experiments were similar to those which induced maximum repair in the other cell lines.

#### *Levels of Enzymes Involved in DNA Synthesis*

To determine whether the inability to incorporate thymidine into DNA was due to a general decrease in the enzymes of DNA metabolism or was merely a consequence of the loss of a single crucial enzyme, the activity of DNA polymerase  $\alpha$ , DNA polymerase  $\beta$  and thymidine kinase were measured in extracts of retinal tissue of different embryonic ages. The activity of both DNA polymerase  $\alpha$  (calculated as the difference between total polymerase activity and  $\beta$  polymerase activity [Fig. 3]) and thymidine kinase decreased in parallel by at least 20-fold between 6 and 10 days. The enzyme levels then remained almost constant from 10 to 14 days. DNA polymerase  $\beta$ , experimen-

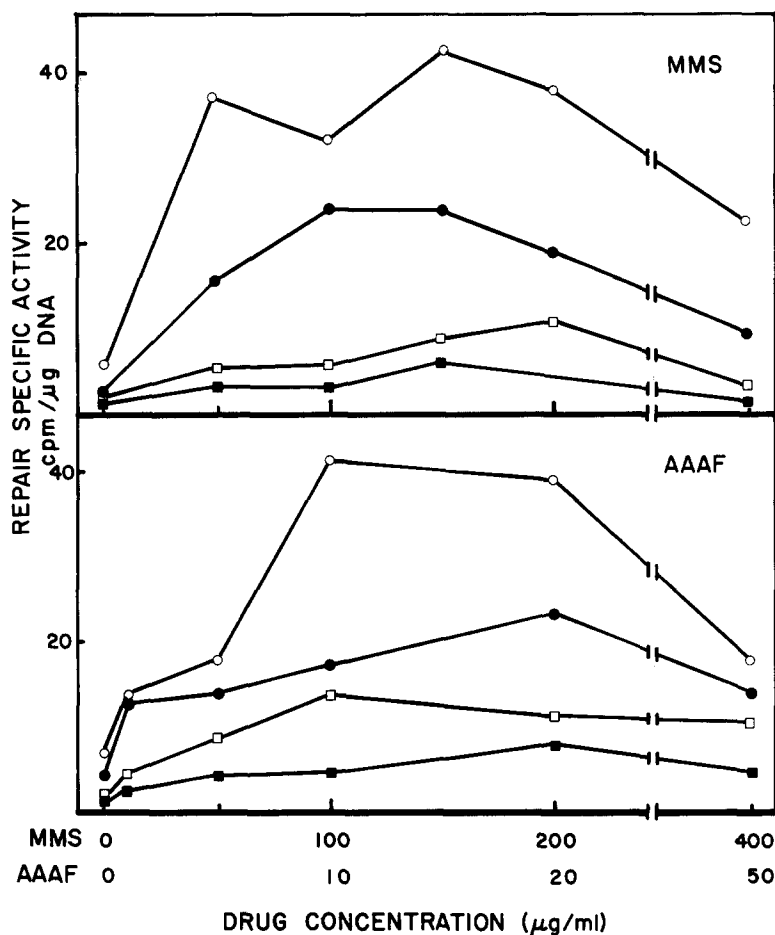


FIGURE 1 The ability to carry out DNA excision repair in primary cultures of chick neural retinal cells as a function of developmental stage of the retina. Cell suspensions were obtained by trypsinization of 8-, 10-, 13- and 15-day retinas; the cells were maintained, in Medium 199 containing 10% FCS, at 37°C on a gyratory shaker. Following pretreatment for 30 min at 37°C with 10 μM cytosine arabinoside, the cells were treated with MMS or AAAF at the concentrations shown, for 60 min in the presence of [<sup>3</sup>H]thymidine (10 μCi/ml, 11–17 Ci/mmol) and 10 μM AraC. The cells were then harvested, washed, and lysates were prepared for chromatography on BND cellulose. ○, 8 day cells; ●, 10 day cells; □, 13 day cells; ■, 15 day cells. Each point represents the mean of two determinations.

tally measured as the residual DNA polymerizing activity resistant to inhibition by NEM, declined only 3-fold between days six and fourteen (Fig. 3). Assays of extracts of 6-day cells in the presence of extracts of 14-day cells indicated that the age-dependent decrease in DNA polymerizing capacity was due to the lack of active enzyme and not to the presence of a diffusible inhibitor (Table II). In order to provide a standard of comparison, we determined that the specific activity of thymidine kinase in extracts of 6-day cells was approximately  $\frac{2}{3}$  of that found in the human lymphoblastoid cell line Raji.

#### *Effect of Stimulation of DNA Synthesis on the Levels of DNA Repair*

Neural retina cells from 14-day chick embryos can be stimulated to synthesize DNA by dissociating the cells with trypsin and culturing them in monolayer cultures. The incorporation of [<sup>3</sup>H]TdR into cultures derived from trypsinized 14-day cells increases about 1,000-fold in the 7 days after plating (Fig. 4a). Although the exact kinetics of stimulation varied from experiment to experiment, the overall pattern was an approxi-

mately 100-fold increase in the specific radioactivity in cells given a 60-min pulse of [ $^3\text{H}$ ]TdR by the third to fourth day after initiation of the cultures, and a further 10-fold increase within the next 3 to 4 days. This DNA synthesis is sensitive to inhibition by AraC, and we assume it to be replicative and nuclear.

Measurements of DNA repair induced by concentrations of MMS (150  $\mu\text{g}/\text{ml}$ ) and AAAF (20  $\mu\text{g}/\text{ml}$ ) which elicited maximal repair in retinas from 6–15-day embryos were carried out on successive days on monolayer cultures initiated from 14-day retina cells. Despite the large increase in replicative DNA synthesis in these cultures (see above), there was no corresponding increase in the levels of DNA repair (Fig. 4*b*). The specific radioactivity of the DNA from untreated cells observed in the NaCl eluate from BND-cellulose increased somewhat after day 4. This increased level probably reflects the increased level of replicative synthesis, even though inhibition by AraC was always equal to, or greater than, 97% in these cases.

Allowing for the expected variation in the experimental determination of very low specific activities of repair, none of the determinations of

drug-induced repair gave results which differed from the controls ( $p = 0.3$ ), with the possible exception of the AAAF-induced repair on day 7 of culture ( $p \leq .05$ ). That is, with the possible exception of AAAF-repair at later times, these replicating cell populations are deficient in detectable DNA excision repair.

#### *Development of a Cell Line from Differentiated Retinal Cultures*

After the initial plating of a single cell suspension of 14-day retinocytes, many of the cells tended to cluster into loose clumps. Within 3 to 4 days, the clumped cells had spread out radially around the original foci. The cells divided and after about ten days the culture had become an almost confluent monolayer with the original foci no longer evident. Following subculture by diluting the cells 1:8 after trypsinization, the cell clumping and outgrowth from cell clumps were repeated, and a similar state of confluence was attained in about 3 weeks. The cells in these cultures have been described as large epithelioid retinocytes (LER) (11, 12) and may be of retinogial origin. Their overall morphological charac-

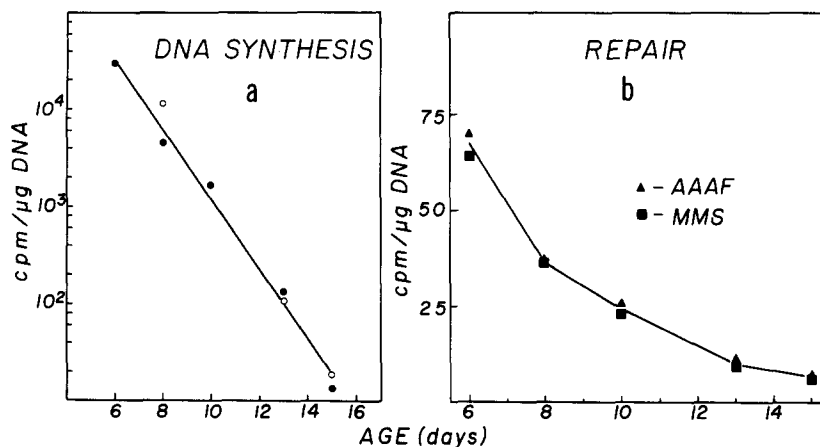


FIGURE 2 (a) The decline in ability to perform replicative DNA synthesis during development of the chick neural retina. Cell suspensions were pulse-labeled with [ $^3\text{H}$ ]thymidine (10  $\mu\text{Ci}/\text{ml}$ , 11–17  $\text{Ci}/\text{mmol}$ ) for 60 min at 37°C and prepared for BND-cellulose chromatography. The specific activity of incorporation was calculated from the absorbance (260 nm) and the radioactivity of the material eluting with 1.0 M NaCl buffer. Closed and open circles refer to separate determinations on different preparations of cells. (b) The decline in the ability to carry out DNA excision repair in primary cultures of chick retinal cells as a function of the developmental stage of the retina. Specific activities of repair induced by various concentrations of MMS and AAAF were determined as in Fig. 1. The ordinate represents the maximum specific activity of repair induced by drug treatment, minus the observed specific activity in the absence of the drug. The abscissa represents the age of the embryo from which retinal cell suspensions were prepared. Each point is the mean of at least two determinations.

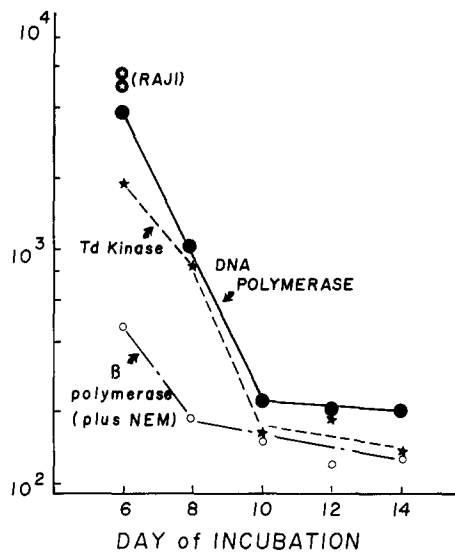


FIGURE 3 Variations in the activity levels of enzymes involved in DNA metabolism in developing chick retina. Extracts of retinal tissue removed from embryos at the ages indicated were assayed for total DNA polymerizing activity, NEM-resistant DNA polymerase and thymidine kinase as described in Materials and Methods. All activities are calculated from the range over which the incorporation of radioactive precursors were linear with extract concentration. In the presence of 1.0 mM NEM, DNA polymerase  $\alpha$  is inhibited to about 90% and DNA polymerase  $\beta$  to about 12% (15). These partial inhibitions were included in the calculation of DNA polymerase  $\beta$  activity. Ordinate: pmol/ $\mu$ g protein; polymerase, 30 min incubation; kinase, 10 min incubation.

teristics resemble those described by Varon et al. (30) for cells in cultures derived by trypsinization from adult rabbit brain and identified as neuroglia (Fig. 5).

The overall capacity of the cell population to incorporate [ $^3$ H]thymidine was monitored by pulse-labeling of separate cultures at daily intervals throughout the initial culture phase. The specific activity (cpm/ $\mu$ g) of the DNA extracted from pulse-labeled cultures increased over the period of the first six days and reached a plateau value after 6–7 days (Fig. 4a). Following subculture of nearly confluent monolayers, the cells continued to incorporate [ $^3$ H]TdR at the same specific activity as the plateau level in the original culture. These data are consistent with a process of selection for replicating cells yielding a population which exhibits a stable rate of proliferation.

### Excision Repair in the Cell Line

Treatment of established cell cultures after the second passage (two subcultures by trypsinization) with either AAAF or MMS did not induce the levels of repair activity expected on the basis of the observed relationship between proliferative ability and repair capacity in the primary differentiating retinas (Table III). About one third of the expected specific repair activity was observed after treatment with 20  $\mu$ g/ml of AAAF. No significant repair activity was ever observed after treatment with MMS (150  $\mu$ g/ml). The value of the specific activity (cpm/ $\mu$ g DNA) in the 1.0 M NaCl eluate from the BND-cellulose eluate of lysates from MMS-treated cells was found to be below the level of the control untreated cells. We assume that this reflects an increased inhibition of DNA replication by MMS itself which, in the absence of repair synthesis, lowers the specific activity of this frac-

TABLE II  
Absence of Inhibitory Activity for DNA Polymerase in Extracts of Later-Stage Chick Embryo Retinas

	[ $^3$ H]dTTP incorporation cpm/ $\mu$ g protein
Day 6 extract	273.2 241.6
Day 10 extract	22.5 22.6
Day 12 extract	18.1 19.8
Day 14 extract	21.2 23.8
Day 6 extract + day 10 extract	215.5 246.8
Day 6 extract + day 12 extract	228.7 213.3
Day 6 extract + day 14 extract	275.8 223.4

Extracts of 6-day retina were assayed for total DNA polymerase activity as described in Materials and Methods. The incorporation of [ $^3$ H]dTTP into acid-insoluble material was calculated for day 6 extract (18  $\mu$ g protein) assayed alone and in the presence of extracts of day 10 (80  $\mu$ g protein), 12 (92  $\mu$ g protein) or 14 (90  $\mu$ g protein) retinas. Incorporation is calculated per microgram of protein from the 6-day extract.

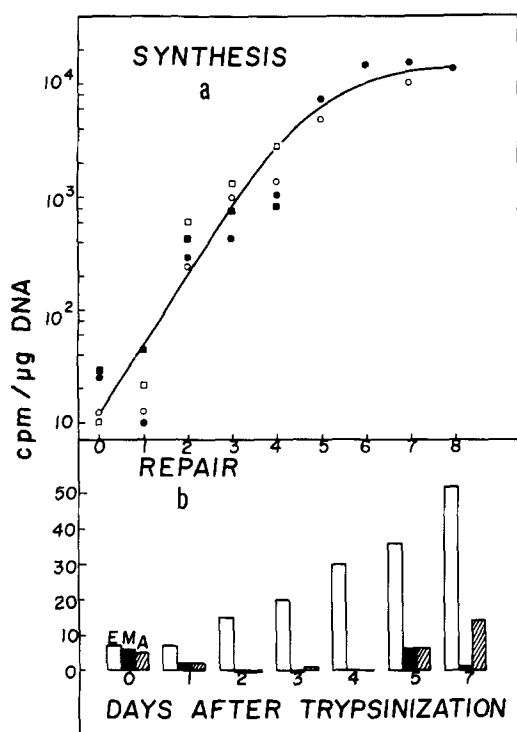


FIGURE 4 (a) Stimulation of DNA synthesis in monolayer cultures of cells derived from trypsinized 14-day embryonic retina. Retinas removed from 14-day embryos were trypsinized, and the resultant single-cell suspension was seeded into plastic tissue culture flasks (25 cm<sup>2</sup>) containing 5 ml of Medium 199. After incubating at 37°C for 2 h to allow cell attachment, the medium was replaced with 5 ml of Medium 199 containing 10% FCS and pulse-labeled with 10  $\mu$ Ci/ml [<sup>3</sup>H]thymidine (11–17 Ci/mmol) for 60 min at 37°C. Separate cultures were pulsed each day under the same conditions. In longer-term experiments, the cells were fed on day 4 of culture. After washing the cells, lysates were prepared for BND-cellulose chromatography. The specific activity (cpm/ $\mu$ g) of the DNA was calculated from the absorbance (at 260 nm) and the radioactivity eluted with 1.0 M NaCl buffer. Circles and squares (closed and open) represent data from four different experiments, and each point is the mean of two determinations. Determinations on replicate cultures within each single experiment agreed to within 15%. (b) DNA excision repair in cells from 14-day embryonic retina in which DNA synthesis was stimulated by dissociation with trypsin. Single cell suspensions were prepared from 14-day embryonic retinas and seeded into plastic tissue culture flasks (25 cm<sup>2</sup>) containing Medium 199 plus 10% FCS (Day 0). Excision repair was measured in replicate cultures treated with either MMS (150  $\mu$ g/ml) or AAAF (20  $\mu$ g/ml), as described in the legend to Fig. 1. Two cultures received

tion by further decreasing the residual DNA chain elongation that removes incorporated labeled thymidine from the growing point region. The rate of DNA synthesis in these cultures is comparable to that in 6-day retina cells, which accounts for the relatively high specific activity in the controls even at high levels of inhibition by AraC.

#### Extent of Reaction with DNA

The choice of drug concentrations used in the determination of repair activity in these experiments was based on two criteria. First, as mentioned above, these concentrations induce the maximal rate of repair in a wide variety of different types of cell; and second, the inhibition of DNA synthesis which we assume is related to the extent of reaction of the agent with DNA is closely similar in human lymphoblastoid cells (Raji) and the retina cell line (Table IV) which developed upon further culture. This suggests that the observed deficiency in repair represents a true defect in the repair process and cannot be attributed to differential permeability of cells to MMS resulting in different levels of reaction with DNA.

The question of reactivity was studied directly in experiments in which cells were reacted with radioactively labeled AAAF or MMS. The cells were then lysed and the DNA was purified by equilibrium banding on CsCl gradients. The DNA peak from each gradient was pooled and the radioactivity and absorbancy were determined. The levels of reaction were calculated and are shown in Table V. We found no significant differences in the reactivity of MMS with the DNA from the different cell types examined, indicating that lack of drug accessibility was not the cause of our failure to observe MMS-induced repair synthesis

no drug (controls); another culture was used to measure the overall incorporation of [<sup>3</sup>H]TdR in the absence of both AraC and drug treatment. Repair measurements were performed on the days (after plating the cells) indicated in the abscissa. The culture medium was changed on day 4. The ordinate represents the specific activity of repair (cpm/ $\mu$ g DNA) induced by drug treatment after subtraction of control values. M, repair (cpm/ $\mu$ g DNA) induced by 150  $\mu$ g/ml MMS; A, repair (cpm/ $\mu$ g DNA) induced by 20  $\mu$ g/ml AAAF; E, expected repair based on the DNA synthetic capacity of the cell population estimated by comparison with Fig. 2.



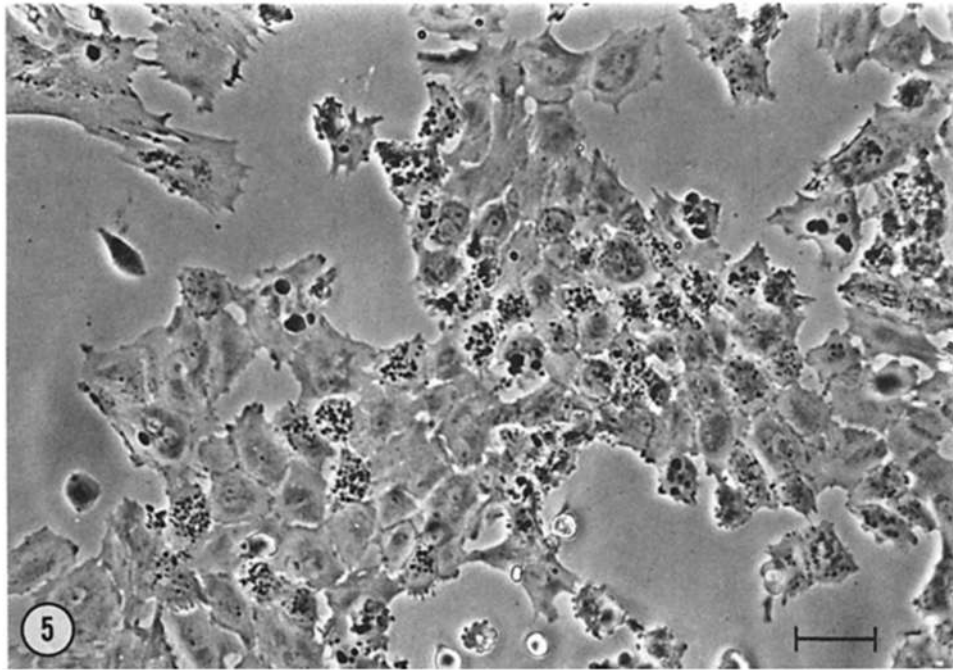


FIGURE 5 Phase photomicrograph of a living culture of cells derived from trypsinized 14-day retinas.  $\times 200$ . Scale bar, 50  $\mu\text{m}$ .

TABLE III  
DNA Repair in Established Retinal Cell Cultures

Experiment number	Specific activity			Repair specific activity (mean treated-mean control)	
	Control	MMS	AAAF	MMS	AAAF
	<i>cpm/<math>\mu\text{g}</math></i>				
1	27.7	8.5		-14.2	
	16.9	7.6		(+60)	
2	27.9	17.9	49.9	-7.9	20.6
	23.6		42.8	(+60)	(60)

Inhibition by AraC = 98%; expected values shown in parentheses. Long-term retinal cell cultures derived from trypsinized 14-day retinas were used after the second passage. To the monolayers, in 10 ml of Medium 199 plus 10% FCS, was added cytosine arabinoside (10  $\mu\text{M}$ ) and, after 30-min incubation at 37°C, MMS (150  $\mu\text{g}/\text{ml}$ ) or AAAF (20  $\mu\text{g}/\text{ml}$ ) and [ $^3\text{H}$ ]TdR (10  $\mu\text{Ci}/\text{ml}$ , 11–17 Ci/mmol) were added. Repair was allowed to proceed for a further 60 min at 37°C. After removal of the radioactive medium, the cells were washed and lysed *in situ* in 5 ml of SSC containing 0.2% SDS. The specific activity of repair and the percentage inhibition of DNA synthesis were determined as described in the legend to Table I.

in either late-stage retinas or the outgrown monolayer cultures. Retinal cells also reacted with AAAF to give approximately equal numbers of adducts; indeed, the 14-day cells which are deficient in repair at all concentrations (Fig. 1) were, if anything, more reactive than 8-day cells. The high reactivity of the outgrown monolayer cells is surprising. It may be that the reactivity towards cells in monolayers differs from the reactivity towards cells treated at high concentration in suspension when a compound with a short half-life is used. It may also be that this particular cell type has high intrinsic reactivity. We are investigating these possibilities.

## DISCUSSION

Previous studies on rodent and human cells (13, 26) have shown that there is a correlation between the ability of a cell population to carry out the reactions of excision repair and its capacity for replicative DNA synthesis. During the embryonic development of the chick neural retina, DNA synthesis decreases about 1,000-fold between 6 and 15 days of embryogenesis. This decrease is accompanied by an approximately 10-fold reduction in the rate of DNA excision repair.

Regan and Setlow (24) concluded that human cells repair damage via two reaction modes differing in the size of the repair patch. "Long patch" repair (about 100 nucleotides) is characteristic of the response to damage induced by ultraviolet light or AAAF; "short patch" repair (3-4 nucleo-

tides) is observed after treatment with X-rays or MMS. This difference in repair mode is reflected in the initial rates of repair synthesis as measured by the BND cellulose method, using the human lymphoid cell line, Raji (26). A difference in rates of repair for MMS and AAAF was not observed with chick retinal cells at any stage of development. At least two explanations might account for the identity in repair rates: (a) Chick cells have a limited ability to incorporate exogenous thymidine, and therefore repair rates measured after AAAF treatment do not reflect the true values because of saturation of the thymidine incorporation system; or (b) the repair systems in chick cells differ in some way from those in Raji cells.

The level of thymidine kinase in the 6-day embryo retinocytes is about two thirds that found in Raji cells. A 60-min pulse of [<sup>3</sup>H]thymidine results in incorporation of radioactivity by the retinocytes to give a specific activity of  $2 \times 10^4$  cpm/ $\mu$ g DNA. Under similar conditions, Raji cells incorporate thymidine to a specific activity of  $1.08 \times 10^4$  cpm/ $\mu$ g of DNA. Since the chick nucleus has only about one half as much DNA as the human, we would expect chick cells to incorporate thymidine to a specific activity of about twice that of human cells in a 1-h pulse, if the rate of DNA chain elongation is identical in both cells. We determined a ratio of 1.85. The comparison indicates that there is no limitation in the ability of the retina cell to utilize exogenous thymidine as a

TABLE IV  
The Inhibition of DNA Synthesis in Outgrown Monolayer Cultures of Retinal Cells by MMS

MMS concentration $\mu$ g/ml	Control incorporation of [ <sup>3</sup> H]TdR (%)	
	Retinal cell line	Raji lymphoblastoid cell line
0	100	100
20	61.5	75
50	24	23
100	7.1	7.8
200	3.5	3.1

Long-term cultures derived from trypsinized 14-day retinas were used after the second passage. To each culture, in 10 ml of Medium 199 containing 10% FCS, was added MMS to the final concentrations shown and, following an incubation of 60 min at 37°C, [<sup>3</sup>H]TdR (10  $\mu$ Ci/ml, 15 Ci/mmol). After a further incubation of 60 min at 37°C, the radioactive medium was removed, and the cells were washed in PBS and finally lysed *in situ* in 5 ml of SSC containing 0.2% SDS. The total acid-insoluble tritium was determined for each lysate. The inhibition data for the human lymphoblastoid line, Raji, are taken from (25).

TABLE V  
Extent of Reaction of AAAF and MMS with DNA

Cell type	Compound			
	AAAF		MMS	
	conc $\mu$ g/ml	Adducts per $10^8$ daltons DNA	conc $\mu$ g/ml	Adducts per $10^8$ daltons DNA
8 day retina	100	37.0	100	53.9
14 day retina	100	66.5	100	41.8
Outgrown monolayer (second passage)	100	1,798.2	100	46.6
Raji	100	49.5	40	16.7

$10^7$  freshly prepared 8-day and 14 day retina cells in 0.5 ml of Medium 199 containing 10% fetal calf serum were treated with either [<sup>14</sup>C]MMS or [<sup>14</sup>C]AAAF to a final concentration of 100  $\mu$ g/ml. After incubation at 37° for 60 min (MMS) or 30 min (AAAF), cells were washed free of unreacted drug by centrifugation, lysed, and digested with pronase and RNase. Monolayer cultures of the outgrown cell line were treated with the same concentration of drug in 2 ml of medium (MMS) or 5 ml of medium (AAAF) for the same times as the suspension cultures. Lysates were prepared and digested. After shearing the lysates (5  $\times$ , 20 gauge needle), the volumes were adjusted to 8 ml with SSC and the density to 1.70 by the addition of solid CsCl. The resulting solutions were banded for 40 h at 40,000 rpm and 20° in the Beckman Ti50 rotor. After fractionation, the regions of absorbance at 260 nm from each gradient were pooled, the DNA content of the pooled fraction was calculated, and the acid-insoluble radioactivity was determined. The gradient fractions immediately adjacent to the absorbance peak were also pooled and served as a background measurement for both absorbance and radioactivity. The values for Raji cells are taken from (26).

DNA precursor, even for replicative synthesis, as compared to Raji cells. Therefore, the observed inability of the 6-day retinal cells to repair AAAF damage to DNA at the same rate as Raji cells, a process requiring less phosphorylated thymidine than does replication, cannot merely be a consequence of the inability to phosphorylate [<sup>3</sup>H]TdR. By the same argument, we rule out the possibility that the lower AAAF-induced repair activity is due to a greater thymidine pool size in retinal cells. The reduction in repair capacity observed in the chick retinocytes between 10 and 14 days is unlikely to be due simply to a deficiency in thymidine phosphorylation or DNA polymerase, since the activities of these enzymes remain relatively constant over this period.

Chick neural retina cells differ therefore from the human cell line, Raji, in their relative response to MMS and AAAF. However, the significance of the equivalent MMS- and AAAF-induced maximal repair rates is not clear. The parallel decline in the rates of both MMS-induced and AAAF-induced repair over the 6–15-day period of embryogenesis implies, however, that some common factor is involved.

In this context, the data from cell cultures established from suspensions of 14-day embryo retina cells allow some distinctions to be made. In these cultures, DNA replication is reinitiated. If the ability to carry out the excision repair of damaged DNA were coupled to the ability of cells to replicate DNA, then an increase in the overall replicative activity of the cell population should have resulted in an increased capacity for excision repair of both types of damage as observed with human lymphocyte populations (26). However, the 1,000-fold increase in replicative DNA synthesis in the cell culture established from retinas of 14-day embryos was not accompanied by the expected increase in DNA repair capability.

It is clear that factors other than the replicative state of the cells determine repair capability even in genetically competent cells. We assume that, as is the case in other systems in which DNA synthesis is experimentally stimulated (liver regeneration, lymphocyte stimulation by mitogens), the levels of DNA polymerases, nucleoside kinases and other enzymes involved in the metabolism of DNA are all increased when replication is reinitiated in retina cells (22, 23). The reduced level of DNA repair after MMS- treatment must therefore reflect the deficiency of an enzyme (or enzymes) specifically involved in the repair of alkylation

damage as distinct from other enzymes involved in both the replication and repair of DNA.

This inference is supported by the repair studies with the cell line propagated from suspensions of 14-day retinocytes. We were unable to detect even a limited capacity for repairing MMS-induced damage, which shows that the cell line derived from the originally post-replicative retina cells is defective in at least one step of the pathway for the excision repair of MMS-induced damage to DNA. It is not clear whether the defect extends to the AAAF repair pathway.

The exact identity of the cells in the long-term cultured line established from suspensions of 14-day embryonic neural retina cells is unknown; we consider them to be of retinogial origin for the following reasons: (a) The chick embryonic neural retina is avascular, which rules out the possibility that cultures derived from it contain fibroblasts, endothelial cells or blood cells. (b) There is no evidence for the presence of significant numbers of cells with typical neuronal morphology. (c) The population comprises predominantly large epithelioid cells (LER cells (11)), and their morphological characteristics are similar to those described for neuroglia in cell cultures (30). (d) Nakai and Okamoto (20) reported that no cellular elements other than glial cells are found in long-term cultures of neural cells derived from an initially avascular neural tissue. (e) The normally low rate of glial cell proliferation in the brain is considerably enhanced by tissue damage (1), and trypsinization of the neural retina to dissociate the cells may be analogous to tissue damage.

Fig. 5 is a photomicrograph of a long-term retina cell culture which had been trypsinized and replated twice. About 50% of the cells are pigmented. Also present in the culture are small numbers of lens cells (not shown). The possibility that both pigmented cells and lens cells in these cultures are derived from a common (LER, retinogial) precursor cell has been considered (10, 11).

There are certain suggestive correlations between the ability of cells to repair DNA damage induced by alkylating agents and carcinogenesis in neural tissue. *N*-ethyl-*N*-nitrosourea induces a high proportion of brain tumors in fetal rats during the period of proliferative activity in the brain (2). Goth and Rajewsky (8) have demonstrated that the alkylation product, O<sup>6</sup>-ethyl guanine, is removed from brain tissue at a much lower rate than in other organs. The O<sup>6</sup>-alkyl guanines have been

implicated as being particularly significant in mutagenesis in both prokaryotic (16) and eukaryotic (18) systems. The observations suggest that cells in neural tissues have a defective excision repair system for alkylation damage and that failure to repair DNA may lead to reactions involved in carcinogenesis.

The predominant tumors of neural tissue in man are of glial origin (7), and the alkylation-induced nervous system tumors in neonatal rats are predominantly cerebral gliomas (29). Our results raise the question of whether the deficiency in DNA repair found in neural retina and in cell cultures derived from it might not apply in general to neuronal cells and (in particular) to retinogial cells. The production of gliomas could then be thought of as a consequence of the inability of this cell type to repair damage to its DNA in conjunction with its capacity to proliferate in response to tissue damage.

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