both DNA and protein increase rapidly between 17 and 20 days of gestation. During the same period the activity of both lactase and alkaline phosphatase in the small intestine is also found to increase steadily. Taken together, the results indicate that growth and maturation of the fetal small intestine are extremely rapid during the last part of gestation.

In recent years, considerable evidence has accumulated to suggest that EGF, a mitogenic polypeptide, promotes growth of the digestive tract in both young and adult rats[6-8, 19, 20]. Our present observation that addition of EGF to the organ culture system containing fetal small intestinal explants stimulates the rate of synthesis of both DNA and protein suggests that (a) EGF promotes small intestinal cell proliferation, and (b) is independent of other hormone(s) or growth-promoting factor(s). The observed increment in incorporation of ^{3}H-valine and ^{3}H-thymidine into protein and DNA of small intestinal explants by EGF could not be attributed to an increased entry of the precursors. This interpretation is based on the observation that there was no significant difference in acid-soluble radioactivity (for both ^{3}H-valine and ^{3}H-thymidine) between the basal and EGF-treated samples.

Although under the present experimental condition EGF induced small intestinal growth as evidenced by increased DNA and protein synthesis, it had no affect on the functional maturation of the tissue. This interpretation is based on the observation that addition of EGF to the organ culture system produces no apparent change in either lactase or alkaline phosphatase activity when compared with the corresponding basal control. Such an observation is analogous to what we[8] as well as Dembinski and Johnson[9] have earlier observed in suckling rats. In contrast, Calvert et al.[11] have demonstrated that in mice administration of EGF during the later stages of pregnancy significantly stimulates alkaline phosphatase and trehalase activities in the fetal small intestine without affecting DNA and protein content in the tissue. However, since the observation was made after in vivo administration of the peptide to the mothers, it is difficult to assess whether the aforementioned findings are due to EGF by itself or secondary to EGF administration.

In conclusion, the present data demonstrate that whereas EGF promotes growth related processes of the fetal small intestine, it does not affect the functional maturation of the organ.

1 Acknowledgment. The project was supported by grants from the Veterans Administration Research Service. The authors wish to thank Dr M.C. Geokas, Chief, Department of Medicine, Veterans Administration Medical Center, Martinez, CA, for providing us with excellent laboratory facilities and for his encouragement in this study.

2 Gregory, H., Nature *257* (1975) 325.
3 Cohen, S., J. biol. Chem. *237* (1962) 1555.
4 Cohen, S., Proc. natn. Acad. Sci USA *72* (1963) 1.
5 Sundell, H., Pediat. Res. *9* (1975) 371.
6 Dembinski, A.B., and Johnson, L.R., Endocrinology *116* (1985) 90.
7 Feldman, E.J., Aures, D., and Grossman, M., Proc. Soc. exp. Biol. Med. *159* (1978) 400.
8 Majumdar, A.P.N., J. Pediat. Gastroent. Nutr. *3* (1984) 618.
9 Hirata, Y., and Orth, D.N., Endocrinology *105* (1979) 1381.
10 Carpenter, G., Science *210* (1980) 199.
11 Calvert, R., Beaulieu, J.-F., and Menard, D., Experientia *38* (1982) 1096.
12 Kaysen, G.A., Majumdar, A.P.N., Dubick, M.A., Vesenka, G.D., Mar, G., and Geokas, M.C., Am. J. Physiol. *249* (1985) F523.
13 Wannemacher, R.J. Jr, Banks, W.L. Jr, and Wunner, W.H., Analyt. Biochem. *11* (1965) 320.
14 Bradford, M., Analyt. Biochem. *72* (1976) 248.
15 Dahlqvist, A., Analyt. Biochem. *22* (1968) 99.
16 Bessey, O.A., Lowry, O.H., and Brock, M.A., J. biol. Chem. *164* (1946) 321.
17 Moog, F., in: Textbook of Gastroenterology and Nutrition in Infancy, p. 139. Ed. E. Lebenthal. Raven Press, New York 1981.
18 Klein, R.M., and McKenzie, J.C., J. Pediat. Gastroent. Nutr. *2* (1983) 10.
19 Johnson, L.R., and Guthrie, P.D., Am. J. Physiol. *238* (1980) G45.
20 Konturek, S.J., Radecki, T., Brzozowski, T., Piastucki, I., Dembinski, A., Dembinska-Kiec, A., Zmuda, A., Gryglewski, R., and Gregory, H., Gastroenterology *81* (1981) 438.

0014-4754/86/080950-03$1.50 + 0.20/0

Competition between retinol and 3,4-didehydroretinol for esterification in crude pigment epithelial cell fractions

A.T.C. Tsin

Division of Life Sciences, The University of Texas at San Antonio, San Antonio (Texas 78285, USA), 6 September 1985

Summary. The membrane fraction of the retinal pigment epithelium (RPE) of the frog *(Rana pipiens)* catalyzed the esterification of tritiated retinol to retinyl esters. This esterification reaction was inhibited in the presence of 3,4-didehydroretinol.
Key words. Retinol; 3,4-didehydroretinol; esterification; pigment epithelium; frog.

Many animals possess both rhodopsin (a retinal based visual pigment) and porphyropsin (a 3,4-didehydroretinal based visual pigment) in their retinas[1-6]. In these animals, both retinyl and 3,4-didehydroretinyl esters are also found in their retinal pigment epithelium (RPE)[7-11] located adjacent to the retina. The composition of rhodopsin/porphyropsin in the retina is similar to the composition of retinyl/3,4-didehydroretinyl esters in the pigment epithelium in some species[8, 12] but not in others[10, 13, 14]. In order to fully understand how the composition of visual pigments changes in response to light[10, 13, 15-18], temperature[10, 13, 18-20] and other factors[10, 21-23], it is necessary to study the pathway whereby retinol and 3,4-didehydroretinol are stored and utilized by the eye. Retinoids in the eye are found mainly in the RPE as retinyl esters of long chain fatty acids such as palmitic and stearic acids[24-26]. The present report shows how retinol and 3,4-didehydroretinol compete with each other for the esterification to palmitic acid resulting in the formation of retinyl and 3,4-didehydroretinyl palmitate.

The membrane component of the RPE/choroid from four frogs (*Rana pipiens*, average weight: 12 g) was obtained by homogenizing freshly removed RPE/choroid in amphibian Ringer solution (111.2 mM NaCl, 2.0 mM KCl, 1.1 mM $CaCl_2$ and 2.4 mM $NaHCO_3$; pH = 7.3) and centrifugation (100,000 × g; for 1 h). The membrane fraction (pellet) was then resuspended in 6 ml of Ringer solution and divided into six equal portions (tubes 1–6). Only tritiated all-*trans* retinol (0.2 nmol, 14×10^6 dpm, in 30 µl of ethanol) was added to the first tube. Tritiated retinol (quantity and radioactivity same as in tube 1) was mixed with cold (non-radioactive) all-*trans* retinol (50 nmol) and the mixture was dried by nitrogen and the residue re-dissolved in 30 µl of ethanol. The mixture was added to the second tube and this procedure was repeated for the third test tube. Similarly, tritiated retinol (quantity and radioactivity same as in tube 1) was mixed with cold all-*trans* 3,4-didehydroretinol (50 nmol) and the mixture redissolved in 30 µl of ethanol and added to the fourth tube (and this procedure was repeated for the fifth tube). Tritiated retinol

Esterification of tritiated all-*trans* retinol by the frog RPE in the presence of all-*trans* retinol, all-*trans* 3,4-didehydroretinol and all-*trans* retinal

Tube number	Radioactivity of retinyl palmitate (cpm)	Level of inhibition by cold retinoids
1. [^{3}H] retinol	166,000	0
2. [^{3}H] retinol + cold retinol	847	196
3. [^{3}H] retinol + cold retinol	928	178
4. [^{3}H] retinol + cold 3,4-didehydroretinol	848	196
5. [^{3}H] retinol + cold 3,4-didehydroretinol	995	167
6. [^{3}H] retinol + cold retinal	165,000	0

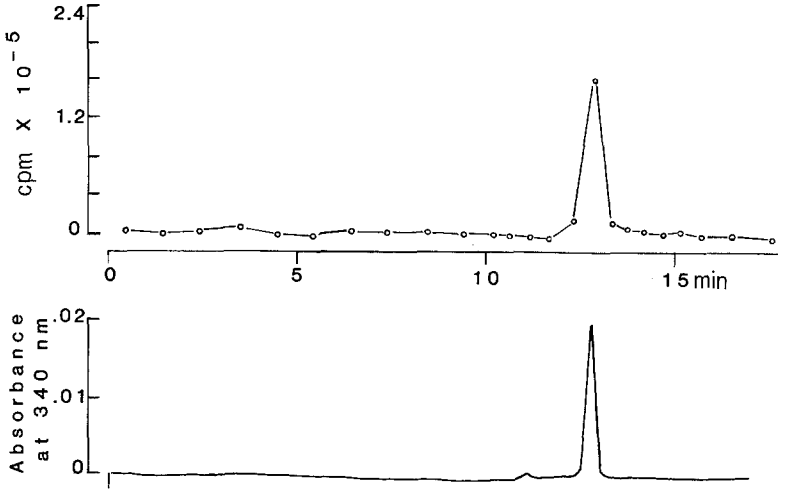
(quantity and radioactivity same as in tube 1) was mixed with all-*trans* retinal (50 nmol) and the mixture redissolved in 30 µl of ethanol. This mixture was then added to the sixth tube to study whether molecular dilution by another non-radioactive retinoid may exert an influence on the esterification of tritiated retinol. All tubes were incubated at room temperature for 2.5 h before centrifugation (100,000 × g, for 1 h). The pellet was extracted with acetone and retinyl esters in the extract were isolated by an alumina column and then analyzed by HPLC (high-performance liquid chromatography, 11 & 27). The ester fraction eluted from the alumina column contained only (radioactive) retinyl palmitate (fig.). with no detectable quantity of any other retinyl esters. The identity of this retinyl ester was established by co-chromatography with authentic retinyl palmitate[27]. The retinoid origin of this ester was further established by saponification and then co-eluting with all-*trans* retinol[11]. Retinyl palmitate was then collected from the HPLC eluant, mixed with Scintilene (Fisher Scientific) and measured for the level of radioactivity in a liquid scintillation counter (counting efficiency: 59%).

The labeling of retinyl palmitate suggests that the esterifying enzyme in the frog RPE was membrane-bound (table, tube 1), which is in agreement with results of others[28,29]. Presence of the cold (non-radioactive) all-*trans* retinol (tubes 2 and 3) resulted in a significant reduction (about 200 times) of radioactivity of retinyl palmitate as compared to that in tube 1. This level of inhibition is in agreement with the molar ratio between tritiated and cold retinol (1:250). As the cold retinol competed with tritiated retinol for the same active site on the esterifying enzyme, the quantity of radioactive retinyl palmitate (as a result of the esterification of tritiated retinol) was then proportionately reduced. As shown in the table (tubes 4 and 5), the addition of cold all-*trans* 3,4-didehydroretinol also inhibited the formation of radioactive retinyl palmitate at a level similar to that exhibited by cold all-*trans* retinol (tubes 2 and 3). All-*trans* retinal (250 molar excess) did not inhibit the labeling of retinyl palmitate by exogenous tritiated retinol.

These results suggest that frog pigment epithelium contains an enzyme which can esterify exogenous retinol to retinyl palmitate. Because 3,4-didehydroretinol possesses the same ability as retinol to inhibit tritiated retinol for esterification (table), these retinols probably possess a similar affinity for the active site of the esterifying enzyme. In a separate study, it was established that these crude RPE membrane fractions can also esterify all-*trans* 3,4-didehydroretinol to all-*trans* 3,4-didehydroretinyl palmitate[30], suggesting that the same active site on this enzyme is responsible for the esterification of both retinol (to retinyl palmitate) and 3,4-didehydroretinol (to 3,4-didehydroretinyl palmitate).

Acknowledgments. This research was supported by grants from the National Science Foundation (BNS 82-03064) and the National Institutes of Health (RR-08194 and GM07717). I thank Hoffman La-Roche (Nutley, New Jersey) for the generous gift of tritiated retinoic acid and all-*trans* 3,4-didehydroretinol.

1 Wald, G., J. gen. Physiol. *19* (1935) 351.
2 Wald, G., J. gen. Physiol. *22* (1939) 775.
3 Dartnall, H. J. A., and Lythgoe, J. N., Vis. Res. *5* (1965) 81.
4 Schwanzara, S. A., Vis. Res. *7* (1967) 121.
5 Lythgoe, J. N., in: Handbook of Sensory Physiology, vol. 7, pt. 1, p. 604. Ed. H. J. A. Dartnall. Springer-Verlag, New York 1972.
6 Liebman, P. A., in: Handbook of Sensory Physiology, vol. 7, pt. 1, p. 481. Ed. H. J. A. Dartnall. Springer-Verlag, New York 1972.
7 Wald, G., J. gen. Physiol. *22* (1939) 391.
8 Bridges, C. D. B., and Yoshikami, S., Vis. Res. *10* (1970) 1333.
9 Bridges, C. D. B., Vis. Res. *15* (1975) 1311.
10 Tsin, A. T. C., and Beatty, D. D., Expl Eye Res. *29* (1979) 15.
11 Tsin, A. T. C., Alvarez, R. A., Fong, S.-L., and Bridges, C. D. B., Vis. Res. *24* (984) 1835.
12 Reuter, T. E., White, R. H., and Wald, G., J. gen. Physiol. *58* (1971) 351.
13 Tsin, A. T. C., and Beatty, D. D., Expl Eye Res. *30* (1980) 143.
14 Tsin, A. T. C., Beatty, D. D., Bridges, C. D. B., and Alvarez, R. A., Invest. Ophthal. Vis. Sci. *24* (1983) 1324.



HPLC profile of retinyl palmitate extracted from tube 1. The eluant of the HPLC was collected in 1-ml fractions (0–10 min) and 0.5-ml fractions (10–15 min). These fractions were then measured for radioactivity to produce the profile of radioactivity. The major peak in both absorbance and radioactivity profiles is all-*trans* retinyl palmitate. Column: 5 µm Ultrasphere; flow rate: 1 ml/min; eluant: 0.2% dioxane in n-hexane; detection: 340 nm.

15 Dartnall, H.J.A., Landers, M.R., and Munz, F.W., in: Progress in Photobiology, p.203. Eds B. Christensen and B. Buchmann. Elsevier, Amsterdam 1961.
16 Bridges, C.D.B., Vis. Res. *10* (1979) 1315.
17 Allen, D.M., Vis. Res. *11* (1971) 1077.
18 Tsin, A.T.C., Vis. Res. *19* (1979) 1269.
19 Allen, D.M., and McFarland, W.N., Vis. Res. *13* (1973) 1303.
20 Tsin, A.T.C., and Beatty, D.D., Science *195* (1977) 1358.
21 Beatty, D.D., Vis. Res. *9* (1969) 855.
22 Beatty, D.D., Vis. Res. *12* (1972) 1947.
23 Cristy, M., Gen. comp. Endocr. *23* (1974) 58.
24 Futterman, S., and Andrews, J.S., J. biol. Chem. *239* (1964) 4077.
25 Alvarez, R.A., Bridges, C.D.B. and Fong, S.-L., Invest. Ophthal. Vis. Sci. *20* (1981) 304.
26 Bridges, C.D.B., Fong, S.-L., and Alvarez, R.A., Vis. Res. *20* (1980) 355.
27 Bridges, C.D.B., and Alvarez, R.A., Meth. Enzym. *81* (1982) 463.
28 Berman, E.R., Horowitz, J., Segal, N., Fisher, S., and Feeney-Burns, L., Biochim. biophys. Acta *630* (1980) 36.
29 Fong, S.-L., Bridges, C.D.B., and Alvarez, R.A., Vis. Res. *23* (1983) 47.
30 Tsin, A.T.C., J. expl Zool. *238* (1986) 311.

0014-4754/86/080952-03$1.50 + 0.20/0

Fluorescence response of acridine orange to changes in pH gradients across liposome membranes

A.A. Cools and L.H.M. Janssen

Department of Pharmaceutical Chemistry, State University of Utrecht, Catharijnesingel 60, NL-3511 GH Utrecht (The Netherlands),
4 April 1985

Summary. The influence that changes in proton distribution have on the fluorescence of acridine orange was examined using negatively charged liposomes. Our results indicate that at least two mechanisms are involved: distribution of the probe between the internal aqueous phase of the liposomes and the outside medium, and binding of the probe to the liposome membranes.
Key words. Acridine orange; fluorescent probe; pH gradient; fluorescence quenching; liposomes.

Fluorescent amines are useful tools to monitor pH gradients across energy transducing and model membranes[1,2]. It is assumed that these compounds, being weak bases, can move freely across membranes in their unprotonated form and, if a pH gradient is established, will accumulate in their protonated form on the side of the membranes where the pH is lower, in accordance with the pH gradient. The distribution of an ideal probe between the inside and the outside aqueous phases of vesicles or liposomes due to a pH gradient can be estimated directly from the quenching of its fluorescence[2]. However the exact mechanism of quenching is not yet fully understood[3].

The question of why acridine orange in the presence of pH gradients does not behave like an ideal probe has been the subject of some discussion in the literature. Some authors have interpreted the fluorescence quenching of acridine orange in the presence of energized membranes largely as a binding phenomenon[4,5]. Lee and Forte however, as a result of their experiments with gastric microsomes, postulated that when acridine orange is used as a fluorescent probe both distribution between aqueous phases and binding may be involved at the same time[6]. Along the same line, we show evidence in the present paper for a mixed mechanism for fluorescence quenching of acridine orange by using a membrane model system, i.e. negatively charged liposomes.

Materials and methods. Dipalmitoyl-phosphatidylcholine (DPPC) and dicetyl-phosphate (DP) were purchased from Sigma Chemical Company. Acridine orange (AO) was obtained from Merck. Monensin was a gift from Eli Lilly Research Laboratories and was added from an ethanolic solution.

Liposomes were prepared according to the method described by Bangham et al.[7]; 20 mg DPPC and 2 mg DP were dissolved in 5 ml chloroform-methanol (2:1). The organic solvents were evaporated in a rotary evaporator. The remaining phospholipid film was suspended either in 1 ml solution containing TrisCl (100 mOsM), MOPS/KOH (10 mOsM) or in 1 ml solution containing NaAcetate (100 mOsM), MOPS/KOH (10 mOsM) at 55°C; mOsM was used instead of mM for the purpose of equal tonicity inside and outside the liposomes. From here NaAcetate will be referred to as NaAc. The liposomes were used after an 1-h period of equilibration.

Fluorescence measurements were performed with a Perkin-Elmer (type 204) fluorimeter. A front surface light cell (angle 45°), fixed in a light cell holder, equipped with a thermostat (temperature 55°C), was used. The light cell was filled either with 1 ml solution containing TrisCl (100 mOsM), MOPS/KOH (10 mOsM), AO (50 µM) or with 1 ml solution containing NaAc (100 mOsM), MOPS/KOH (10 mOsM), AO (50 µM) or with 1 ml solution containing NaCl (100 mOsM), MOPS/KOH (10 mOsM), AO (50 µM). The fluorescence was adjusted to 100% with 490 nm as the excitation wavelength and 530 nm as the emission wavelength. Then 50 µl liposomes were added and the fluorescence quenching was recorded, without corrections for dilution.

Results and discussion. From figure 1 it can be seen that AO fluorescence increases steadily with increasing AO concentration until a concentration of about 0.1 mM is reached. At higher concentrations the fluorescence decreases. The experiments described below were therefore performed using 50 µM AO. Anomalous fluorescence changes, due to concentration quenching, are not to be expected at this concentration. When 50 µl liposomes, which had been prepared in TrisCl buffer (pH 7.4),

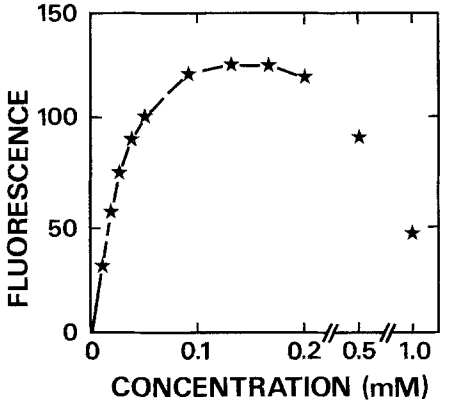


Figure 1. Fluorescence (in arbitrary units) of acridine orange in TrisCl buffer (pH = 7.4) as a function of acridine orange concentration.