7. Slyshenkov, V. S., Moiseenok, A. G. and Wojtczak, L., *Free Radical Biol. Med.*, 1996, **20**, 793–800.
8. Cross, C. E., Halliwell, B., Borish, E. T., Pryor, W. A., Ames, B. N., Saul, R. L., McCord, J. M. and Harman, D., *Ann. Intern. Med.*, 1987, **107**, 526–545.
9. Fridovich, I., *J. Biol. Chem.*, 1989, **264**, 7761–7764.
10. Taniguchi, N., *Adv. Clin. Chem.*, 1992, **29**, 1–59.
11. Przedborski, S. and Schon, E. A., *Nature Genet.*, 1998, **13**, 99–100.
12. Chelack, W. S. and Petkau, A., *Biochim. Biophys. Acta*, 1981, **660**, 83–90.
13. James, J. E., *Caffeine and Health*, Academic Press, London, 1991.
14. Kihlman, B. A. (ed.), *Caffeine and Chromosomes*, Elsevier, New York, 1977.
15. Selby, C. P. and Sancer, A., *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 3522–3525.
16. Kesavan, P. C., *Curr. Sci.*, 1992, **12**, 791–797.
17. Devasagayam, T. P. A. and Kesavan, P. C., *Indian J. Exp. Biol.*, 1996, **34**, 291–297.
18. Kesavan, P. C., Trasi, S. and Ahmad, A., *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.*, 1973, **24**, 581–587.
19. Kesavan, P. C. and Powers, E. L., *Int. J. Radiat. Biol.*, 1985, **48**, 223–233.
20. Farooqi, Z. and Kesavan, P. C., *Mutat. Res.*, 1992, **269**, 225–230.
21. George, K. C., Hebbar, S. A. and Kesavan, P. C., *J. Radiol. Protection*, 1999, **19**, 171.
22. Abraham, S. K., *Mutat. Res.*, 1991, **262**, 109–114.
23. Sanz, F., Lopez-deBrinas, Rodriguez, J. and Manaut, M., *Quant. Struct-Act. Relat.*, 1994, **13**, 281–284.
24. Devasagayam, T. P. A., Kamat, J. P., Mohan, H. and Kesavan, P. C., *Biochim. Biophys. Acta*, 1996, **1282**, 63–70.
25. Chatterjee, S. R., Srivastava, T. S., Kamat, J. P. and Devasagayam, T. P. A., *Mol. Cell. Biochem.*, 1997, **166**, 25–33.
26. Sun, M. and Zigman, S., *Anal. Biochem.*, 1978, **90**, 81–89.
27. Sajan, M. P., Satav, J. G. and Bhattacharya, R. K., *Toxicol. Lett.*, 1995, **80**, 55–60.
28. Kamat, J. P., Sarma, H. D., Devasagayam, T. P. A., Nesaretnam, K. and Basiron, Y., *Mol. Cell. Biochem.*, 1997, **170**, 131–138.
29. El-Nahas, S. M., Mattar, F. E. and Mohamed, A. A., *Mutat. Res.*, 1993, **301**, 143–147.
30. Neriishi, K., in *Vitamin E – Its Usefulness in Health and in Curing Diseases* (ed. Mino, M.), Japanese Science Society Press, Tokyo, 1993, pp. 187–194.
31. Ben-Amotz, A., Rachmilevich, B., Greenberg, S., Sela, M. and Wesler, Z., *Radiat. Environ. Biophys.*, 1996, **35**, 285–288.
32. Sarma, L. and Kesavan, P. C., *Int. J. Radiat. Biol.*, 1993, **63**, 759–764.
33. Isoherranen, K., Peltola, V., Laurkainen, L., Punnonen, J., Laihia, J., Ahotupa, M. and Punnonen, K., *J. Photochem. Photobiol. B. Biol.*, 1997, **40**, 288–293.
34. Rice-Evans, C. and Burdon, R., *Prog. Lipid Res.*, 1993, **32**, 71–110.
35. Dean, R. T., Fu, S., Stocker, R. and Davies, M. J., *Biochem. J.*, 1997, **324**, 1–18.

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Cell population growth during the formation of the chick eye lens

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The eye lens, a radially symmetrical and autonomously growing organ, contains dividing cells in the epithelium while fibre cells do not divide. Between day 7 and day 15 of chick embryogenesis, the annular pad extending from the peripheral epithelium is formed which acts as a reservoir of proto-differentiated fibres that are then transferred to the fibre area. Around the optical axis, a wave of apoptosis is generated in terminally differentiated fibre cell nuclei. We have determined cell numbers in the epithelium, the annular pad and fibre compartments and the data fit a logistic model the best, indicating a saturating growth. The growth rates for various lens compartments change with time.

THE evaginating optic vesicle comes in contact with the head ectoderm and induces the lens placode[1] that invaginates to form the lens vesicle[2]. The primitive lens contains prospective lens fibres in the inner hemisphere and prospective epithelium in the outer hemisphere[3,4] and becomes an autonomously growing organ. The prospective fibre cells stop dividing[4], enter a G_{01} phase[5] and synthesize lens crystallins[6–8]. The prospective epithelium contains dividing cells[4]. Progressively, the peripheral epithelial cells establish a distinct germinative zone[9,10] beyond which a reservoir, the annular pad, of non-dividing proto-differentiated fibres appears. These cells also contain fibre-specific δ crystallins[8] and are transferred to the fibre area throughout the life span[5,10]. The lens offers the best model for programmed cell death or apoptosis as elongated terminally differentiated primary fibre cells, located along and around the optical axis, exhibit a temporally and spatially specific pattern of nuclear pycnosis and loss[5]. The apoptotic lens cell nuclei lose DNA *in situ*[5], undergo DNA strand scission[11] liberating free 3^1OH ends detected as initiators *in situ* for calf thymus terminal deoxynucleotidyl transferase[12–15]. In both native[16,17] and 2-D (native and denaturing) agarose gels, it was shown[18] that fibre nuclear DNA breaks down and forms a DNA ladder respecting the polynucleosomal structure of chromatin, and loses the histone H-1 (refs 17,19). The fibre cell bodies devoid of nuclei and packed with crystallins, pile around the optical axis. Throughout the life span, the lens cell

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population grows wherein the epithelial cell progeny contributes to both epithelial and fibre cell populations; the latter via the transfer compartment or the annular pad.

Earlier modelling[20] revealed that, between 2½ and 12 days of development, both epithelial and fibre cell populations grow exponentially and establishment of the lens cell lineage is not a clonal event. Recently, a geometrical model based on shape changes and lens volume growth, has been published[21], but it is inadequate to describe the cell population growth dynamics of the lens. We now estimate cell population sizes in various lens compartments and fit these to 4 different growth models. We show that the logistic growth model fits best for the data on 50 lenses distributed over the entire embryonic period and 15 days after hatching.

Fifty chicken lenses of 3–20 days embryogenesis and 15 days after hatching were dissected in Tyrode, fixed in Carnoy, embedded in paraffin and serially sectioned (5 μm thick) along the plane perpendicular to the equator and parallel to the optical axis[20]. Sections were stained in Mayer's hemalum and mounted in Permount or Eukitt.

To estimate cell numbers, we have divided the lens in 5 major compartments. (i) The central epithelium, (ii) the peripheral epithelium, (iii) the annular pad (AP) with proto-differentiated fibers, (iv) nucleated fibres (NFB) harbouring the differentiating nucleated fibre cells, and (v) lens fibre cells showing pycrotic nucleic[5] in phases I and II (PYC). The geometrical coordinates of the region containing non-nucleated (NNC) fibres can be measured but not the cell numbers. The top one-third AP also contains dividing cells and the bottom third contains elongated proto-differentiated fibres; AP was subdivided into the outer AP (OAP), middle AP (MAP) and inner AP (IAP). The number of cells in the central and peripheral epithelium, AP, NFB and PYC was counted and corrected using Abercrombie's formula[22] as described[20].

Until the 5th day of embryogenesis, the entire epithelial cap contains dividing cells. After 7 days, the AP is progressively formed at the outer rim of the peripheral epithelium and the dividing cell population becomes restricted to a germinative zone common to the peripheral epithelium and the top (or outer third) of the AP[10] or OAP. Thereafter, central epithelial cells exhibit decreasing proliferative activity and become non-dividing[10], but maintain the division potential[23]. Along the lens equator, the AP joins fibre cells where elongated and proto-differentiated IAP cells, present as a single row, turn around their long axis by 90° and become confluent with cortical lens fibres. The middle third pad (MAP) is a pseudo-stratified region between the IAP and OAP. In the lens fibre area, only NFB are seen till the 8th day of development, and, then, those along the optical axis, begin to exhibit nuclear pycnosis and this region is termed as PYC. The pycnosis starts at the centre and spreads as a wave radially outwards[5]. By

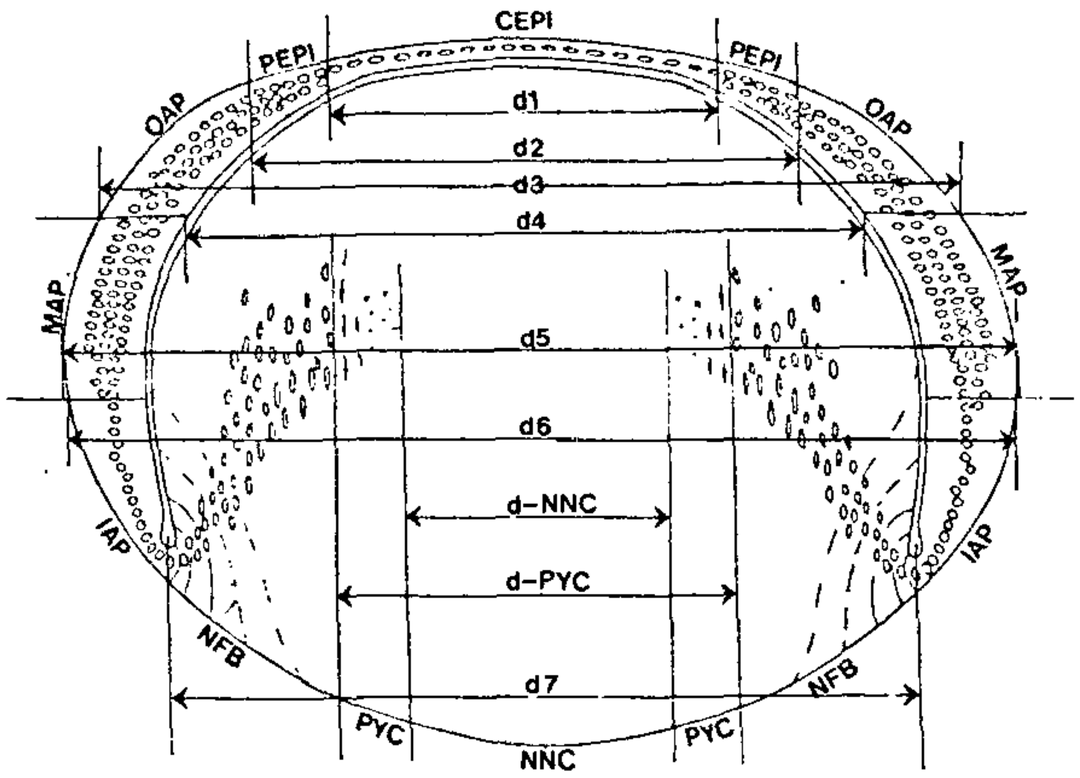

Figure 1. Schematic drawing of a 17-day-old chick lens section showing lens compartments: CEPI, central epithelium; PEPI, peripheral epithelium; OAP, outer annular pad; MAP, middle annular pad; IAP, inner annular pad; NFB, nucleated fibre region; PYC, fibres with pycnotic nuclei; NNC, non-nucleated fibres. Vertical lines show the inner and outer boundaries of lens compartment. Horizontal lines, d1–d7 represent the inner and outer diameters of CEPI, PEPI, OAP, MAP, IAP and NFB. d-PYC and d-NNC are diameters of PYC and NNC compartments, which appear after 8 days and 14 days, respectively of chick embryogenesis. After 14th day d-NNC is subtracted from d-PYC to get the actual radii of PYC zone.

day 13 all cell nuclei close to the optical axis are pycnotic and this zone, after 15 days, becomes non-nucleated, as nuclei are lost *in situ*[5]. Thus, from day 15, we discern three different fibre regions, namely the nucleated fibres in the cortex and bow, fibres with pycnotic nuclei, and non-nucleated fibres (Figure 1).

The geometric coordinates of all compartments of the radially symmetrical lens were measured as before[20]. Chick lens cells are mono-nucleate and the nuclear counts represent cell numbers in each compartment. Assuming that the sections are compressed on a horizontal plane parallel to the equatorial plane, the total cell number in each compartment of the lens is estimated as described earlier[10,20]. However, this method does not allow us to estimate the number of non-nucleated cells.

Cell numbers were plotted against the lens age and the distributions were analysed using linear regression, power function, exponential function and logistic growth function[24,25]. The linear regression formula, $Y = mX + C$, estimates the rate of increment from the slope m and allows direct comparison of growth rates of different compartments. The power function $Y = a(X)^{2\beta}$, used previously[20] to analyse the growth of lens epithelium and lens fibres, estimates the fractional inputs or the growth fraction (β) in the system, whereas a gives the cell population size when the lens is induced. The logistic growth function $Y = [(A_1 - A_2)/(1 + (X \div X_0)^{\theta})] + A_2$ was also applied to the data. The fits would signify that the population first grows rapidly but then the

Table 1. Growth parameters of the developing chick lens

Lens compartments	Linear regression	Power function		Logistic growth function	
	Growth rate–No. of cells per day**	Growth fraction (β)	Cell number at the time of induction**	Saturating cell population size	Lens age (days) at 50% saturation level
Entire lens	1.965×10^4	0.503	2.28×10^4	7.8×10^5	19
Epithelium (EPI)	1.59×10^3	0.48	2.7×10^3	5.7×10^4	17
Annular Pad (AP)	9.66×10^3	0.6	7.31×10^3	6.7×10^5	38
DIV (EPI + OAP)	6.6×10^3	0.7	2.75×10^3	3×10^5	25
RES (MAP + IAP)	4.6×10^3	0.46	5.51×10^3	1.8×10^5	17
Fibre region (NFB + PYC)*	8.42×10^3	0.49	1.34×10^4	2.6×10^5	14

*Based on cell numbers excluding those for NNC, i.e. after the loss of cell nuclei.

**Data fits are not significant by Runs test ($P > 0.05$).

growth slows down due to a decreased rate of proliferation, cell migration/transfer, and/or cell death, and that the population attains a steady state equilibrium. In this equation, A_1 and A_2 are the initial and final cell numbers, while X_0 is the time when 50% saturation level is attained. The Runs test[25] was applied to data examined for all growth models using the Microcal Origin Version 3.5.

The total lens cell number was estimated by adding those determined for each compartment. Data were plotted against time (Figure 2) and fitted to a linear regression, power function and the logistic growth function. While all fits were statistically significant ($P < 0.005$), only the logistic fit was found to be significant when Runs test, which assesses for the random distribution of data points along the fitted line or curve, was applied ($P < 0.05$). In any case, each function among the three estimates different growth parameters and these are shown in Table 1.

Early modelling studies[20] have revealed that induction and establishment of the lens cell lineage is not a single cell clonal event, and both lens epithelium and fibres grow exponentially up to day 12 of embryonic development. However, the cell division rate of the epithelium is greater than the growth rate of fibres and the AP is established as a reservoir. We have now analysed the growth of epithelium (EPI), the AP (OAP + MAP + IAP), and the fibre (NFB + PYC) compartments up to day 15 after hatching. Both EPI and OAP harbour dividing cells, or those with division potential, and we have analysed the growth of the resultant DIV (EPI + OAP) compartment. The MAP and IAP form the reservoir (RES) of proto-differentiated fibres.

Although the correlation coefficients and P values for linear regression are excellent due to the large data set, the linear function does not fit well the data (Figure 2) beyond 12 day embryogenesis as the dividing lens cell fraction decreases exponentially with time[10] and the input in the system decreases progressively. Furthermore, with the Runs test[25], the fits are not significant.

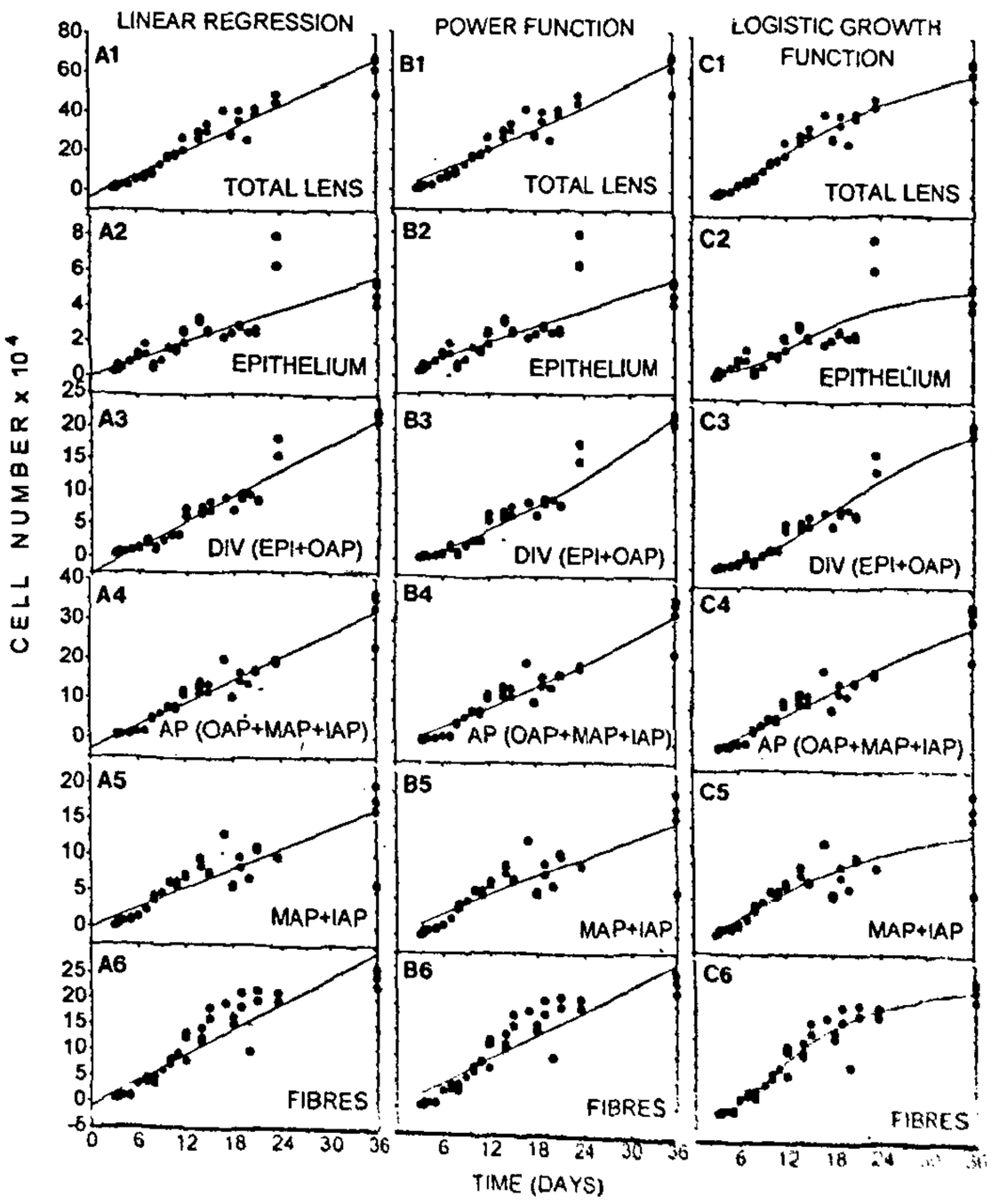

Figure 2. Cell numbers of entire lens and lens compartments are plotted against time (days) and fitted to linear regression (A1 to A6), a power function (B1 to B6) and logistic growth function (C1 to C6). All fits are statistically significant ($P < 0.005$). To note that the best fits are for logistic growth function A6, B6 and C6 offer the best comparison and only C1 to C6 are significant by Runs test.

Nevertheless, using this model we find that both AP and NFB grow fast whereas EPI exhibits the slowest growth rate (Table 1). In contrast, the growth rate is higher in DIV (EPI + OAP), while the fraction RES (MAP + IAP) appears to grow slower (Table 1). Thus, OAP grows much faster than the rest of the AP compartments.

Beyond day 12 of development, the exponential function does not fit the present data (results not shown).

The power function enables estimation of growth fractions of each compartment and, again, we find (Table 1) that DIV has the largest growth fraction of 0.7 while in EPI alone it is 0.48. This is consistent with earlier studies[10] that after 8 days, dividing cells become progressively restricted to the peripheral epithelium and OAP to form the germinative zone. From the increment in the fibre region we conclude that the rate of fibre differentiation is less than in OAP. This is probably why the progeny of DIV, positioned towards the fibre area, accumulates and causes the formation and growth of MAP and IAP (RES) together harbouring proto-differentiated fibres. The estimates of the presumptive cell population sizes of various lens compartments at the time of lens induction derived from the power function vastly exceed the cell number observed at that time so that this method is not applicable for the long-term lens data. Furthermore, the fits are not significant when Runs test was applied.

The lens growth is best described by the logistic growth function (Figure 2, Table 1). The Runs test[24] for a random distribution of data points around the curve fitted best the logistic growth curves alone for all lens compartments. Thus, the growth rates of different lens compartments are constantly changing from the initial exponential to a slower progression during the later part of development. This fits with earlier observations[10] that the dividing cell number increases up to 8 days and decreases thereafter.

During development and ageing, the lens grows in two distinct growth phases. Growth due to cell division is restricted to the DIV compartment, while fibre cell population size grows due to a transfer of non-dividing cells from the AP. In contrast, the growth of the intermediate MAP + IAP (RES) appears to saturate the earliest (Table 1) and then controls the transfer rate to the fibre area. Due to the radial symmetry of the lens, there is no reason to visualize an active transfer or migration of AP cells to the fibre area[10]. We agree that elongated IAP cells can remain at their original location and integrate with the fibre by changing their orientation by 90°. The question of inter-compartment transfer can be settled by applying modelling tools to lenses subjected to ^{3}H-TdR pulse-chase. The present analysis (Table 1) suggests that the fibre cell production reaches 50% saturation level fastest at 15 days which, we feel, is an artefact due to the absence of data on cell numbers in NNC. In any case, to estimate transfer rates, we must determine the number of non-nucleated cells by novel modelling methods and work is continuing in this direction.

1. Spemann, H., in *Embryonic Development and Induction*, Yale University Press, New Haven, 1938.
2. McKeehan, M. S., *J. Exp. Zool.*, 1951, **117**, 31–64.
3. O'Reilly, R. and Meyer, D. B., *Acta Anat.*, 1959, **36**, 20–58.
4. Modak, S. P., Morris, G. and Yamada, T., *Dev. Biol.*, 1968, **17**, 544–561.
5. Modak, S. P. and Perdue, S. W., *Exp. Cell Res.*, 1970, **59**, 43–56.
6. Ikeda, A. and Zwann, J., *Dev. Biol.*, 1967, **15**, 348–367.
7. Piatigorsky, J., *Differentiation*, 1981, **19**, 134–153.
8. Pal, J. K. and Modak, S. P., *Exp. Eye Res.*, 1984, **39**, 415–434.
9. Hanna, C. and Keats, H. C., *Exp. Eye Res.*, 1966, **5**, 111–115.
10. Persons, B. J. and Modak, S. P., *Exp. Eye Res.*, 1970, **9**, 144–151.
11. Modak, S. P., von Borstel, R. C. and Bollum, F. J., *Exp. Cell Res.*, 1969, **56**, 105–113.
12. Modak, S. P. and Bollum, F. J., *Exp. Cell Res.*, 1970, **62**, 421–432.
13. Modak, S. P. and Bollum, F. J., *Exp. Cell Res.*, 1972, **75**, 307–313.
14. Modak, S. P., in *Cell Differentiation* (eds Harris, R., Allin, P. and Viza, D.), Munksgaard Publ., Copenhagen, 1972, pp. 340–342.
15. Modak, S. P., Lever, W. E. and Uppuluri, V. R. R, *Exp. Cell Res.*,.1971, **70**, 465–468.
16. Appleby, D. W. and Modak, S. P., *Proc. Natl. Acad. Sci., USA*, 1977, **74**, 5579–5583.
17. Modak, S. P. and Unger-Ullman, C., in *Differention and Neoplasia, Results and Problems in Cell Differentiation* (eds McKinnel, R. G., DiBernardina, M. A., Blumenfeld, M. and Bergard, R. D.), Springer-Verlag, Berlin, 1980, vol. 11, pp. 178–190.
18. Modak, S. P. and Beard, P., *Nucleic Acids Res.*, 1980, **8**, 2665–2678.
19. Unger-Ullmann, C. and Modak, S. P., *Differentiation*, 1979, **12**, 135–144.
20. Modak, S. P., Uppuluri, V. R. R., Appleby, D. W., Therwath, A. M. and Lever, W. E., *INSERM*, 1976, **60**, 105–112.
21. Marzeck, C. J. and Hendrix, R. W., *J. Theor. Biol.*, 1997, **186**, 349–372.
22. Abercrombie, M., *Anat. Rec.*, 1946, **94**, 239–247.
23. Srinivasan, B. D. and Harding, C. V., *Invest. Ophthalmol.*, 1965, **4**, 452–469.
24. Snedecor, G. W. and Cochran, W. G., *Statistical Methods*, Oxford and IBH, New Delhi, 1967, 6th edn.
25. Gibbons, J. D., in *Nonparametric Methods for Quantitative Analysis*, American Sciences Press, Columbus, Ohio, USA, 1976.

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