Cell cycle analysis of facial mesenchyme in the chick embryo

I. Labelled mitoses and continuous labelling studies

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SUMMARY

Cell cycle parameters were analysed in mesenchyme of the maxillary process and the roof of the stomodeum in the chick embryo from stages 19 through 28. The generation times at stages 24–26 were determined by pulse labelling of embryos with [³H]thymidine, followed by labelled mitosis counts and construction and analysis of percent-labelled mitosis curves employing computer-assisted curve-fitting techniques. The median generation time was approximately 10·6 h in the maxillary process, and 16 h in the roof of the stomodeum; corresponding values for mean generation times were approximately 12·0 and 18·2 h, respectively. Median values for transit times of G_1, S, and G_2 were 2·0, 5·4, and 2·5 h in the maxillary process and 5·2, 6·7, and 2·7 h in the roof of the stomodeum. The distribution of generation times of cells in the roof of the stomodeum, however, appeared to be more heterogeneous than those of cells in the maxillary process.

The percentage of cells which continue to cycle rapidly (i.e. the 'growth fraction') was determined by repeated-labelling experiments with [³H]thymidine in chick embryos from stages 19 through 28. Cumulative labelling of mesenchymal cells in both the maxillary process and roof of the stomodeum approached 100 % at stage 19 but dropped markedly from stage 19 to 25 declining to approximately 60–70 % in the maxillary process, and to 30 % in the roof of the stomodeum. The decline in cell proliferation rates for these regions, determined in previous studies with labelling indices, appears to be a result of the removal of cells from rapidly cycling cell populations into subpopulations which are cycling more slowly and possibly into subpopulations which have become quiescent; the difference in growth rates between these regions could be attributed to the time of appearance and the size of these emerging slow cycling or quiescent subpopulations.

INTRODUCTION

Previous studies indicated that the enlargement of the embryonic facial processes and the development of the primary palate were accompanied by a pattern of decline in rates of cell proliferation throughout the mesenchyme of the embryonic facial region with advancing developmental age (Minkoff & Kuntz, 1977, 1978; Minkoff, 1980). Within the facial primordia (especially in regions where facial primordia attach to each other), rates of cell proliferation were maintained at higher levels compared with adjacent regions or in regions outside of the facial primordia.

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In all of these studies DNA labelling indices were used as an assay for cell proliferation. Although a labelling index is a satisfactory first approximation for evaluating differences in growth rates, a labelling index is a composite of many parameters of the cell cycle (Thrasher, 1966; Cleaver, 1967; Aherne, Camplejohn & Wright, 1977; Steel, 1977). For example, a labelling index represents both the proportion of a cell population that is in the division cycle, as well as the mean generation time of the proliferating compartment. In addition, changes in the age distribution of the population of cycling cells, changes in transit times of individual phases of the cell cycle (and, therefore, the length of S phase relative to the remainder of the cycle), the distribution of intermitotic times of the cycling population, as well as other factors such as cell loss, will be reflected in a labelling index. An alteration in a labelling index seen during development within or among regions could be due to any of the above causes or combinations of them.

For these reasons, a series of experiments employing pulse-labelling, repeated-labelling and label-dilution techniques was performed in order to determine generation times, transit times for phases of the cell cycle, and the fraction of cell populations which was in the division cycle (i.e. the 'growth fraction'). Two areas which had previously been studied with DNA labelling indices, namely the maxillary process and the roof of the stomodeum, were analysed. The developmental ages studied were primarily stages 24 through 26 in the chick embryo, a time during which marked differences in labelling indices had previously been found for these regions (Minkoff & Kuntz, 1978). Cell cycle parameters of mesenchymal cell populations were determined from percent-labelled mitosis curves (PLM) constructed with data derived from chick embryos pulse labelled with [³H]thymidine while estimates of the growth fraction were obtained from continuous-labelling curves.

MATERIALS AND METHODS
PLM studies

Labelling

Chick embryos were incubated, windowed, staged, and labelled with [³H]thymidine (specific activity 20 Ci/mmol, New England Nuclear Co.), by injection of label through a window in the eggshell into the amniotic fluid surrounding the facial region of the embryo. The injection consisted of label diluted with balanced salt solution to a concentration of 3 μCi in 0·1 ml. One hour after injection, label was chased by injection into the amniotic cavity of 1·2 mg of cold thymidine dissolved in 0·5 ml of balanced salt solution. Following the chase, embryos were sacrificed at either half-hour or hourly intervals over a 30 h period by rapid immersion in Bouin's solution.

Histology and autoradiography

After fixation, embryos were dissected, staged (Hamburger & Hamilton,

1951), washed in 50 % alcohol, dehydrated through graded alcohols, embedded in paraffin and sectioned (Humason, 1972) perpendicular to the long axis of the maxillary process. Slides containing cross sections through the middle of the maxillary process and of the roof of the stomodeum were deparaffinized, coated with liquid autoradiographic emulsion (NTB3, Eastman Kodak), dried and stored in light-proof boxes at 4 °C for varying exposure times up to 4 months (Rogers, 1973). Slides were then developed and stained with Weigert's haematoxylin.

Cell counting

Areas were delineated in the maxillary process and the roof of the stomodeum with an ocular grid in a manner analogous to a prior study (Minkoff & Kuntz, 1978). Labelled and unlabelled mitotic cells were counted to determine the percentage of labelled mitoses in these delineated areas at each time point and from the data PLM curves were constructed. At least three or more sections from each embryo were examined and counted; approximately 50–100 mitotic figures were counted in each area for PLM studies and approximately 500 to 1000 cells were counted in each area for determination of mitotic indices using an oil-immersion objective at a magnification of ×1250.

Analysis of PLM data employing computer-assisted curve-fitting techniques was performed by Dr G. G. Steel (Institute of Cancer Research, Belmont, Surrey) using the method of Steel & Hanes (1971). A curve was obtained which appeared to be the best fit to all of the experimental data consistent with their model; from this, the means and their standard deviations, and the median values were obtained for transit times of G_1, S, and G_2. Mean and median values were obtained for generation times as well as the overall distribution of cell cycle times.

Continuous labelling studies

Labelling

Chick embryos were incubated, windowed, staged and labelled with [^{3}H]thymidine by application of label on top of the embryo through a window in the eggshell. Doses ranged from 10 to 20 μCi, depending on the ages of the embryos and the nature of the experiment. Label was diluted with balanced salt solution to approximately 0·1 ml. [^{3}H]thymidine was administered sequentially every $2\frac{1}{2}$ to 3 h over a 15–24 h period (Fig. 3).

Histology and autoradiography

Embryos were fixed in glutaraldehyde and paraformaldehyde (Graham & Karnovsky, 1966), washed in cacodylate buffer for 24 h, dehydrated through graded alcohols to acetone and infiltrated with Epon–acetone mixtures of increasing Epon concentration. They were then embedded in Epon, polymerized

for 3 days at 70 °C (Luft, 1961) and sectioned perpendicular to the long axis of the maxillary process at 2 μm. Slides containing sections through the middle of the maxillary process and of the roof of the stomodeum were processed for auto-radiography as described above. After development, slides were stained with Richardson's stain.

Cell counting

Areas were delineated in the maxillary process and the roof of the stomodeum in a manner described previously (Minkoff & Kuntz, 1978). Labelled and un-labelled cells were counted to determine the cumulative DNA labelling indices. At least three or more sections from each embryo were examined and counted and approximately 500 to 2000 cells were counted in each area for determination of labelling indices using an oil-immersion objective at a magnification of ×1250.

RESULTS

PLM studies

Labelled and unlabelled mitotic figures (late prophase through anaphase) were counted and the percentages of labelled mitoses were used for the construc-tion of PLM curves (Fig. 1). When these data were analysed, a median cell cycle time of 10·6 h and a median S-phase duration of 5·4 h were obtained for mesen-chymal cells in the maxillary process, while in the roof of the stomodeum corres-ponding values were 16 h and 6·7 h, respectively (Table 1). Mean values for the generation time were approximately 12 h (maxillary process) and 18 h (roof of the stomodeum). Median values for G_2 were approximately the same in both regions (2·5 *versus* 2·7 h), while the largest difference between the regions at stages 24–26 was in the transit time of G_1 (median values of 2·0 h in the maxillary process and 5·2 h in the roof of the stomodeum).

The analytical technique employed (Steel & Hanes, 1971; Steel, 1972, 1973, 1977) utilized the form of the labelled mitosis curves to describe the distribution of intermitotic times of the two cell populations. The breadth of the distributions implied by the data is shown in Fig. 2. A much broader distribution is seen in the roof of the stomodeum compared to the maxillary process; in addition, a much larger proportion of cells has relatively long cell cycle times (i.e. over 30 h) in the roof of the stomodeum.

A mitotic index (mid-prophase to early telophase) at stage 24 was determined from autoradiograms of the PLM experiment and, in addition, from histologic sections of cold embryos of the same stages embedded in plastic (Glycol methacrylate cut at 2 μm). A value of 5·11 % was found in the maxillary process and of 2·24 % in the roof of the stomodeum. Assuming that the embryonic cell
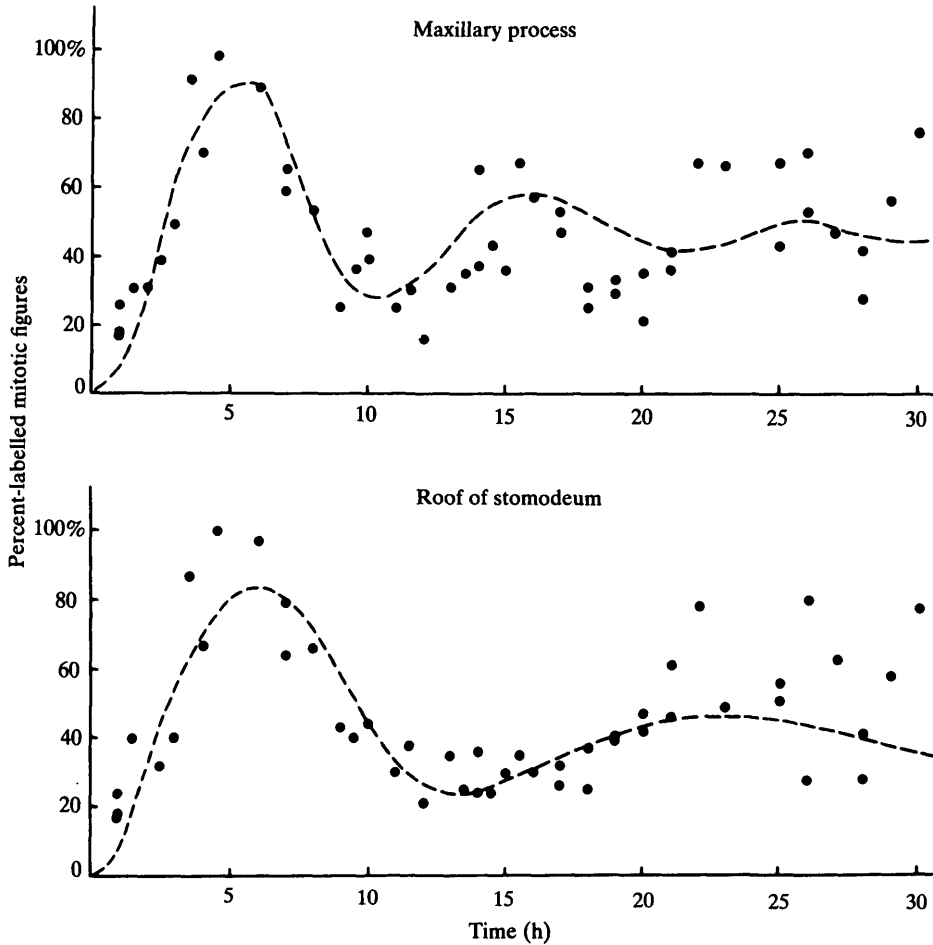
Fig. 1. Percent-labelled mitoses in chick embryos from stages 24–26 after a single injection of [³H]thymidine. A chase with cold thymidine (approximately 1 mg/embryo) was administered 1 h later by injection directly into the amniotic cavity beneath the facial region of the embryo. Embryos were fixed sequentially at half-hour or hourly intervals and processed for autoradiography as described in the text. Each datum point represents one embryo in which approximately 50–100 mitotic figures were counted. PLM curve fitted by the computer program of Steel & Hanes (1971).

populations were growing exponentially and using the formula

$$t_M = I_M t_C / \ln 2 f_G$$

I_M = Mitotic Index
t_C = Generation Time
t_M = Duration of Mitoses
f_G = Growth Fraction

(from Steel, 1977; Aherne *et al.* 1977), mitotic durations of 58 min and 1 h 43 min for the maxillary process and the roof of the stomodeum were obtained. These

Table 1. *Cell cycle analysis of embryonic facial mesenchyme at stages 24–26*

	Region			
	Maxillary process		Roof of the stomodeum	
	[mean (± s.d.)	median]	[mean (± s.d.)	median]
*Transit time G_1 (h)	3·6 ± 5·5	2·0	7·8 ± 8·5	5·2
*Transit time S (h)	5·5 ± 1·0	5·4	6·9 ± 1·6	6·7
*Transit time G_2 (h)	2·9 ± 1·7	2·5	3·5 ± 2·9	2·7
*Median cell cycle time (h)	10·6		16	
Mean cell cycle time (h) Derived from mean transit times of G_1, S and G_2	12		18·2	
Cumulative labelling index (14 h)	60–80 %		20–30 %	

* Optimized parameter derived from computer analysis of curve-fitting technique of Steel & Hanes (1971).
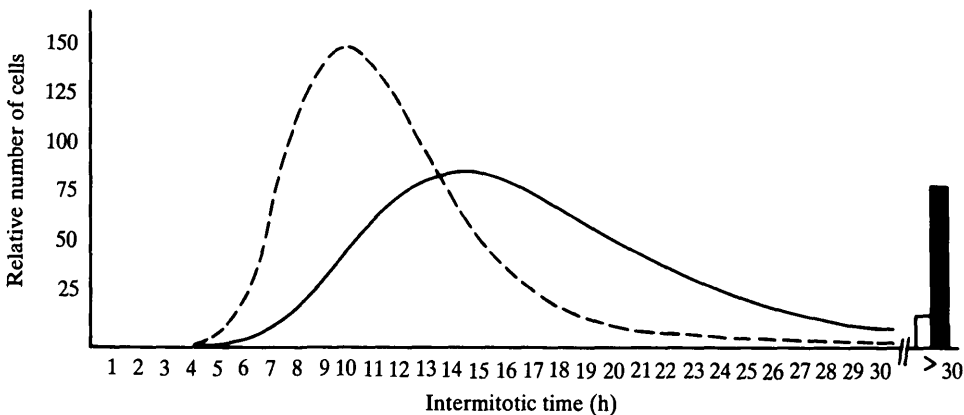

Fig. 2. Distributions of intermitotic times derived from the computer program of Steel & Hanes (1971) corresponding to the labelled mitosis curves for the maxillary process (— — —) and the roof of the stomodeum (———).

values are approximate, however, due to factors such as the width of the mitotic 'window' which was used.

Continuous-labelling studies

Continuous labelling with repeated injections of [³H]thymidine was performed on groups of embryos from stages 19 through 21, stages 24 through 26, and, in more limited experiments with series begun at stage 27 and at stage 28 (see Fig. 3
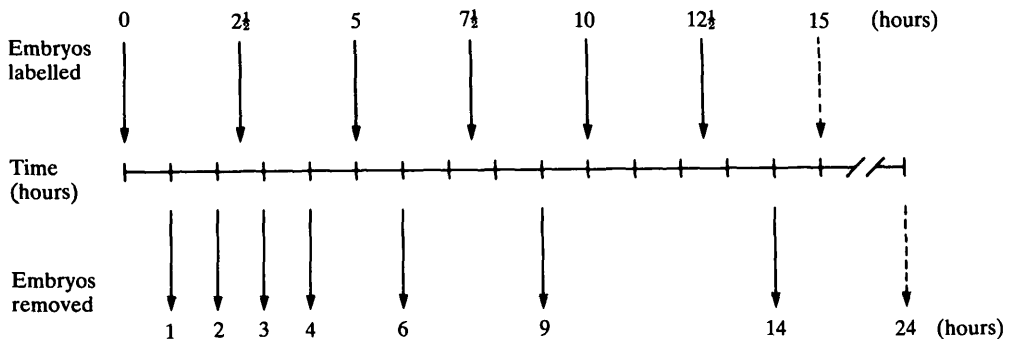
Fig. 3. Outline of protocol for repeated-labelling experiments. Embryos were labelled at the indicated times *in ovo* through a window in the shell and groups of embryos were then removed and fixed at the times shown.

for labelling protocol). When the data from stages 19 through 21 were plotted and analysed (Fig. 4) the fraction of labelled cells continued to increase throughout the 14 h observation period in the maxillary process approaching approximately 80–90 %. The percentage of labelled cells in the roof of the stomodeum also increased throughout the observation period, reaching levels of approximately 70 % after 14 h. By stage 24 through 26, however, a plateau appeared in the curve of the maxillary process between 9 and 14 h at approximately the 60–70 % level while in the roof of the stomodeum the proportion of labelled cells was less than 30 % after 14 h (Fig. 5). Data from the cumulative labelling experiments which were begun later (at stage 27 and at stage 28) displayed patterns similar to those found at stages 24–26. In all cases the cumulative labelling index in the maxillary process rose to approximately 60–70 % at 9 to 14 h while in the roof of the stomodeum it remained at 20–30 %. The difference in the percentage of labelled mesenchymal cells in the two regions was maintained.

In order to confirm the presence of slowly dividing or non-dividing subpopulations, additional experiments were performed utilizing label-dilution techniques. In a limited series of experiments, stage-19 embryos were labelled repeatedly for 9 h and label was then chased with cold thymidine. Embryonic development was allowed to continue in order to dilute the label out of proliferating cells. After 48 h, which corresponded to approximately three to four cell division cycles, the number of grains per nucleus of labelled cells in the maxillary process and in the roof of the stomodeum was determined. The data indicated that the number of grains per nucleus of cells in the maxillary process was reduced to lower values than in nuclei of cells in the roof of the stomodeum, supporting the observations based on cumulative labelling. These preliminary label-dilution experiments were then repeated and extended to later time periods and are the subject of an accompanying report (Minkoff & Martin, 1984).
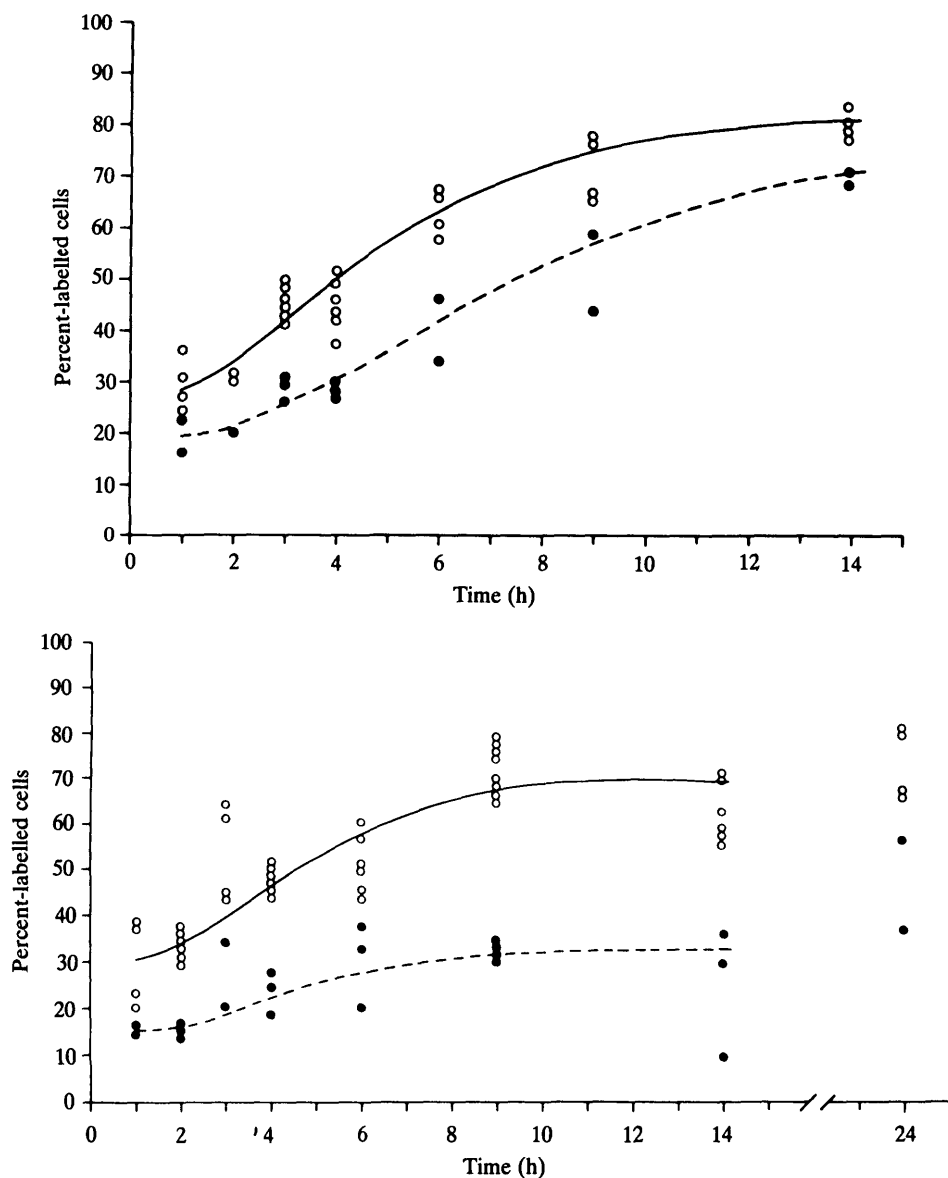
Figs 4, 5. Percentages of labelled cells in the maxillary process (○) and in the roof of the stomodeum (●) after repeated administration of [³H]thymidine (as outlined in Fig. 3). Embryos were fixed and embedded in Epon-812 and processed for autoradiography as described in the text. Each data point represents a total count of 500–2000 cells (from 3 to 12 sections) in each region. Fig. 4, labelling period from stages 19½ through 21½. Fig. 5, labelling period from stages 24 through 26.

DISCUSSION

The results of this study indicated that the differences in DNA-labelling indices of mesenchyme between the maxillary process and the roof of the stomodeum observed previously (Minkoff & Kuntz, 1978) could be attributed, at least in part, to a major difference in the mean generation time of the two cell populations. Differences in generation times in cell populations have generally been found to be due mostly to variation in transit time of G_1 (Cameron, 1971; Aherne *et al.* 1977) and in this study, the major difference in generation times at stage 25 is due to the difference between these regions in G_1. Continuous labelling data as well as the distribution of intermitotic times suggest that the growth fractions of the two cell populations are also different. The accelerated rate of decline of the labelling indices in the roof of the stomodeum, therefore, is probably due to both of these factors, namely differences in the growth fraction as well as mean generation times.

The decline in the growth fraction may represent the removal of cells from a rapidly cycling pool into either a subpopulation which is quiescent (i.e. a viable, non-proliferating but reversible state) or into subpopulations with prolonged durations of the G_1 phase but which continue cycling with extremely long generation times. It is not possible to differentiate between these two alternatives from the cell cycle kinetic data that was obtained.

In a prior study, the labelling indices in the two regions at stages 20–22 were found to be similar, although somewhat higher in the maxillary process (Minkoff & Kuntz, 1978). In the present study, the growth fractions at stage 19–21 were found to be similar. Assuming that both cell populations are growing exponentially and that the age distributions, therefore, are similar, and that cell loss is not a factor, it is probable that the length of the mean generation time of cells in the maxillary process and in the roof of the stomodeum are comparable at stage 19–21. If this is the case, then analysis of all prior labelling experiments (pulse-label, repeated-label, PLM, etc.) would imply that initially, during early stages in the development of the facial primordia, virtually all mesenchymal cells from the regions examined were in the division cycle with a relatively short generation time. As development proceeded, however, one segment of the mesenchymal cell population (primarily in the maxillary process) retained cell cycle characteristics comparable to those of the progenitor cell population while subpopulations appeared (mainly in the roof of the stomodeum) which had longer and a more heterogeneous distribution of generation times.

Because of the possibility that clearance of [^{3}H]thymidine was not efficiently completed during the PLM-pulse-labelling experiments in the stage-24 chick embryos, a cold chase of thymidine was employed as described above. Approximately 1 mg/embryo was the maximum dose used since higher doses may cause cytological abnormalities (Sauer & Walker, 1961). The problem of inadequate clearance during the PLM experiments was also addressed when labelled-mitosis data were analysed. Many reports have demonstrated that the

pattern of a PLM curve may be dependent on the grain count threshold (Wir-sching & Rabes, 1977; Shackney, Ford & Wittig, 1973; Friedman *et al.* 1973; Pickard, Cobb & Steel, 1975), especially in situations where reutilization is suspected (e.g. tumors, regenerating liver) and/or clearance is inefficient. For these reasons, the procedure described by Wirsching & Rabes (1977) was em-ployed prior to the calculations of the percentage of labelled mitoses. After autoradiographic background was obtained and subtracted from data, a broad range of thresholds was tested to eliminate the influence of reutilization and/or inadequate clearance. Repeated analysis of the data indicated that a threshold of eight grains above background reduced these effects to acceptable levels.

In order to determine whether the labelling protocol (and cold thymidine chase) affected the cell cycle during the PLM experiments, a mitotic index was determined on the same slides used for PLM analysis at selected time points throughout the duration of the experiment. No observable effect was detected in either the maxillary process of the roof of the stomodeum from 1 h to 28 h after the onset of labelling with [^{3}H]thymidine (data not shown). It is also unlikely that the multiple application of [^{3}H]thymidine, the time period over which labelling was performed, or the dosages used in the continuous-labelling experiments, had differential effects on cell proliferation. In another study (Minkoff & Martin, 1984), the same labelling protocol had been employed and embryos were grown for periods of 1 week or more after the cessation of labelling. Embryos had developed normally without evidence of growth retardation or of craniofacial abnormalities as a result of the labelling protocol.

In summary, the results of this study suggest that the decline in rates of cell proliferation in embryonic mesenchyme of the facial region is accomplished by the removal of cells from rapidly cycling populations into subpopulations which are cycling more slowly and with a more heterogeneous distribution of genera-tion times and possibly into subpopulations which have become quiescent. Dif-ferences in rates of decline in cell proliferation could be attributed to the time of appearance and size of these emerging subpopulations.

REFERENCES

AHERNE, W. A., CAMPLEJOHN, R. S. & WRIGHT, N. A. (1977). *An Introduction to Cell Popula-tion Kinetics*. Baltimore: University Park Press.

CAMERON, I. L. (1971). Cell proliferation and renewal in the mammalian body. In *Cellular and Molecular Renewal in the Mammalian Body* (ed. I. L. Cameron & J. D. Thrasher). N.Y.: Academic Press.

CLEAVER, J. E. (1967). *Thymidine Metabolism and Cell Kinetics*. Amsterdam: North Holland Publishing Company.

FRIEDMAN, M., NERVI, C., CASALE, C., STARACE, G., ARCANGELI, G., PAGE, G. & ZIPARO, E. (1973). Significance of growth rates, cell kinetics and histology in the irradiation and chemotherapy of squamous cell carcinoma of the mouth. *Cancer* **31**, 10–16.

GRAHAM, R. C. JR. & KARNOVSKY, M. J. (1966). The early stages of absorption of injected horseradish peroxide in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**, 291–302.

HAMBURGER, V. & HAMILTON, H. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49–92.

HUMASON, G. L. (1972). *Animal Tissue Techniques*. San Francisco: W. H. Freeman.

LUFT, J. H. (1961). Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**, 409–414.

MINKOFF, R. (1980). Regional variation of cell proliferation within the facial processes of the chick embryo: a study of the role of "merging" during development. *J. Embryol. exp. Morph.* **57**, 37–49.

MINKOFF, R. & KUNTZ, A. J. (1977). Cell proliferation during morphogenetic change: analysis of frontonasal morphogenesis in the chick embryo employing DNA labeling indices. *J. Embryol. exp. Morph.* **40**, 101–113.

MINKOFF, R. & KUNTZ, A. J. (1978). Cell proliferation and cell density of mesenchyme in the maxillary process and adjacent regions during facial development in the chick embryo. *J. Embryol. exp. Morph.* **46**, 65–74.

MINKOFF, R. & MARTIN, R. E. (1984). Cell cycle analysis of facial mesenchyme in the chick embryo: II. Label dilution studies and developmental fate of slow cycling cells. *J. Embryol. exp. Morph.* **81**, 61–73.

PICKARD, R. G., COBB, L. M. & STEEL, G. G. (1975). The growth kinetics of xenografts of human colorectal tumors in immune deprived mice. *Brit. J. Cancer* **31**, 36–45.

ROGERS, A. W. (1979). *Techniques of Autoradiography*. New York: Elsevier.

SAUER, M. E. & WALKER, B. E. (1961). Radiation injury resulting from nuclear labeling with tritiated thymidine in the chick embryo. *Radiation Res.* **14**, 633–642.

SHACKNEY, S. E., FORD, S. S. & WITTIG, A. B. (1973). The effects of counting threshold and emulsion exposure duration on the percent-labeled mitosis curve and their implications for cell cycle analysis. *Cancer Res.* **33**, 2726–2731.

STEEL, G. G. (1972). The cell cycle in tumours: An examination of data gained by the technique of labelled mitoses. *Cell Tissue Kinet.* **5**, 87–100.

STEEL, G. G. (1973). The measurement of the intermitotic period. In *The Cell Cycle in Development and Differentiation* (ed. M. Balls & F. S. Billett). Cambridge: Cambridge Univ. Press.

STEEL, G. G. (1977). *Growth Kinetics of Tumours*. Oxford: Clarendon Press.

STEEL, G. G. & HANES, S. (1971). The technique of labeled mitoses: analysis by automatic curve-fitting. *Cell Tissue Kinet.* **4**, 93–105.

THRASHER, J. D. (1966). Analysis of renewing epithelial cell populations. In *Methods in Cell Physiology*, vol. II (ed. D. M. Prescott). New York: Academic Press.

WIRSCHING, R. & RABES, H. M. (1977). Grain count threshold dependence of FLM curve pattern and cell cycle parameters of hepatocytes in vivo. *Cell Tissue Kinet.* **10**, 487–496.