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Analysis of the changes in the proportion of clustered labelled cells in epidermis

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Abstract. A new cell kinetic approach is presented from which the duration of the S and G_2 + M phases can be estimated. The technique involves an analysis of the spatial distribution of labelled cells in sections or sheets of epithelium (i.e. an analysis of clustered labelled cells). The technique is largely independent of the absolute number of labelled cells and hence is not influenced by factors which affect the absolute number of labelled cells. The technique is described and experimental data from dorsal murine skin are presented. The technique has also been simulated mathematically so that the phase durations and their variances could be estimated. The advantages of the technique are: (1) it is technically simple; (2) it provides at least two independent estimates of the phase durations; (3) unlabelled cells need not be counted (compare with LI or PLM analysis); (4) it is independent of variations in the absolute yield of labelled cells, and (5) it is applicable if the LI is low and the S phase is short (where the PLM technique tends to fail).

The classical methods of analysing autoradiographs after tritiated thymidine [^{3}H]TdR labelling are to determine the Labelling Index (LI) and the Percentage of Labelled Mitoses (PLM). Both methods evaluate ratios of labelled to total (labelled plus unlabelled) cell counts.

Validity of the LI

The LI represents the number of labelled cells (those in S) divided by the total number of all cells in the proliferative compartment of the tissue under study. If one observes the LI with time, it should start to increase after the first labelled cells pass through the M phase of the cell cycle (see Fig. 1), after which the LI should have doubled, assuming the presence of a post-mitotic compartment. Thus, the LI should, in theory, provide information about the time which the cells spend in the G_2 + M phase (duration of the initial plateau phase) and the length of the S phase (duration of the increase to twice the initial plateau value). However, in practice this information often cannot be extracted. One reason is the biological variance of the S and G_2 + M phase durations, which generally lead to the smooth appearance of experimental curves, such that the end of the G_2 + M phase and the onset of the S phase cannot be identified. A more serious possible problem, however, is presented by

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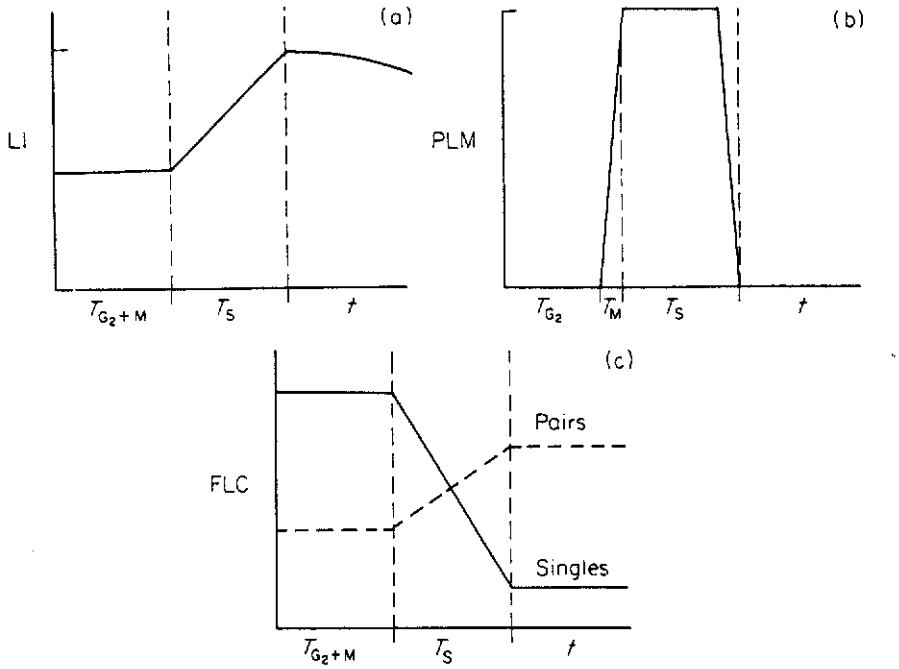

Fig. 1. Theoretical curves for Labelling Index (LI), Percentage of Labelled Mitoses (PLM) and cluster analysis (single/pairs) as fraction of Labelled Cells (FLC). In these schematic curves, no variance is considered. The length of T_S, T_{G2}, T_M is indicated.

[^{3}H]TdR-induced cell death and re-utilization of the label (Potten *et al.*, 1982; Potten & Bullock, 1983; Wichmann & Fesser, 1982; Wichmann *et al.*, 1985). These effects make phase duration estimations difficult or unreliable.

Validity of PLM

A theoretical PLM curve begins at zero immediately after [^{3}H]TdR labelling of S phase cells and increases when the first labelled cells reach the M phase (Fig. 1). The PLM reaches 100% within the duration of the M phase and returns to zero after all labelled cells have left mitosis. The initial plateau describes the length of the G_2 phase and the width of the PLM peak is related to the S phase duration. Due to the biological variance of the cell phase durations, the M phase duration in general cannot be identified. To extract the information on G_2 + M and S phase durations a number of statistical procedures and computer programs are available. In general the PLM method is quite sensitive and is therefore often preferred to the LI.

Nevertheless, it also suffers from some problems. The first is technical: since one has to identify both the label and the mitotic figure of the same cells, the tissue must be only lightly labelled (only a few grains per nucleus). This tends to increase the statistical chance of misclassification of cells which have incorporated [^{3}H]TdR but do not show enough grains per nucleus. The second problem is again related to the possible [^{3}H]TdR-induced artefacts. Early loss of label from initially labelled cells and the re-utilization at different intervals thereafter can lead to broader PLM peaks and even double or triple peaks may appear (Potten *et al.*, 1985, Wichmann *et al.*, 1985), which may make a meaningful estimation of the G_2 + M and S phase duration very difficult.

Analysis of clustered labelled cells

The shortcomings of the LI and PLM methods with respect to the [^{3}H]TdR artefacts mainly arise because both methods compare the number of labelled cells with the total number of

cells. Here we develop a new technique which relates only different cohorts of labelled cells to one another. However, it must be acknowledged that this approach might also have its drawbacks.

Histological preparations, particularly sheets of epithelium, contain information on the spatial distribution or clustering of labelled cells—information that has been largely neglected in the past. We define an *isolated labelled cell as a 'single'* if it is totally surrounded by unlabelled cells. A *'pair'* is defined as *two adjacent labelled cells*, a *'triplet'* as a cluster of three adjacent labelled cells and larger clusters, with more than four labelled cells, *'multiplets'* for convenience.

In order to explain the principles, a hypothetical two-dimensional one-layered epithelium in which all new born cells will remain for a while can be considered. A single cell, labelled at the end of S, will divide after a time T_{G_2+M} and a pair will be formed (Fig. 1). Similarly, a pair will form a quadruplet after passage through G_2 and M phase etc. The total number of singles should remain constant during the G_2 + M phase (Fig. 1). Then it should (theoretically) decrease to zero within an interval which corresponds to the S phase duration, when all singles have become pairs. Similarly, the number of pairs should be constant for G_2 + M. Thereafter it should be influenced by two factors: (a) the 'old' pairs disappear and form quadruplets, and (b) 'new' pairs are formed from the singles after division. The overall proportion will depend on the balance between (a) and (b) (see Fig. 1).

Here, labelling experiments were performed and the changes in the proportions of single and paired labelled cells have been evaluated mathematically.

MATERIALS AND METHODS

Experimental methods

The experiments analysed here were conducted on male BDF1 mice which were 7–8 weeks old. The animals received an intraperitoneal injection of 25 μCi of [^{3}H]TdR (5 Ci/mM) at 03.00 hours or 15.00 hours. Groups of not less than four mice were killed at various times after [^{3}H]TdR administration and samples of skin from the middle of the back were removed. The skin was subjected to acetic acid treatment prior to separation of sheets of epidermis as described elsewhere (Potten & Bullock, 1983). Autoradiographs were prepared (Ilford K5 emulsion, 3–4 weeks exposure).

Scoring

Using a criterion of four grains or more to identify labelled cells, the labelling index and the clustering of labelled cells was determined as follows.

Epidermal sheets

Between 1000 and 2000 basal cells were analysed in the epidermal sheets using an eyepiece Whipple grid (5–10 grids) to determine the labelling index (LI). Using ten grids as reference areas the labelled cells amongst the approximately 2000 basal cells in these grids were analysed for clustering. The number of labelled cells as isolated singles, pairs, triplets, quadruplets and higher clusters was determined, each pair being recorded as two labelled cells, each triplet as three etc. Clusters were scored if more than half of the labelled cells were within the grid. If equal numbers were within and outside the grid, those clusters on two sides of the grid only were recorded. The total number of labelled cells analysed was dependent on the LI value but generally fell withing the range 200–1400 per sample time.

Epidermal sections

The linear clustering of labelled cells in the basal layer (runs of 1 or 2 consecutive labelled cells) was determined from sections of the back skin cut parallel to the long axis of the mouse. Between 100 and 600 labelled cells were analysed per time point which means that about 5000–13,000 basal cells were analysed in the case of the 15.00 hours experiment.

All the results are expressed as the Fraction of all Labelled Cells (FLC) present as clusters of size k (e.g. $FLC_2 = 0{\cdot}20$ means that 20% of all labelled cells appear in pairs). Generally the changes in FLC_k with time are presented. The fraction of labelled cells appearing as singles or pairs are not precisely linked because of the presence of some larger clusters. Consequently we have analysed them as independent measurements.

Mathematical methods

General description of the model

The mathematical approach starts with the assumption that the labelled clusters of type k ($k = 1$: singles; $k = 2$: pairs etc) can conceptually be collected in pools. The pool of labelled singles is denoted by $Y^{(1)}$, the pool of labelled pairs by $Y^{(2)}$ etc. These pools are attributed a cell cycle substructure similar to that employed for the recent analysis of cell kinetics in the epidermal basal layer (Potten *et al.*, 1982, see also Takahashi, 1966, 1968), leading to a subdivision of each pool into n subcompartments $Y_i^{(k)}$ ($i = 1, \ldots, n$). The time development of each of these subcompartments is determined by differential equations of the form:

$$\frac{d}{dt} Y_i^{(k)}(t) = Z_i^{(k)}(t) - \lambda_i^{(k)} \cdot Y_i^{(k)}(t) + f_i^{(k)}(t) \quad \text{for } i = 1, \ldots, n^{(k)} \tag{1}$$

$Z_i^{(k)}(t)$ stands for the transition rate of cells entering into subcompartment i from its precursor subcompartment, while $-\lambda_i \cdot Y_i^{(k)}$ denotes a random disappearance process from subcompartment i to the next subcompartment. As cell death is ignored, all cells leaving subcompartment $i-1$ appeal in subcompartment i:

$$Z_i^{(k)}(t) = \begin{cases} 0 & \text{for } i = 1 \\ \lambda_{i-1}^{(k)} \cdot Y_{i-1}^{(k)}(t) & \text{for } i = 2, \ldots, n^{(k)}. \end{cases} \tag{2}$$

The term $f_i^{(k)}(t)$ in eqn (1) represents the 'cluster creating function'. If it is positive, clusters of the type k and age i are formed; if it is negative, clusters of this type are destroyed. In a two-dimensional layer two mechanisms can generate clusters: mitoses and spatial rearrangement of cells in a tissue. If, for example, a single divides, generally a pair is generated because the daughter cells will be located close together. A pair may be lost (destroyed) if one of the cells divides again (forming a triplet) or if an unlabelled cell migrates and separates the two labelled cells forming two singles or if one of a pair migrates out of the basal layer. In general, clusters of the type k may be added or lost from the pool of k-clusters in any phase of the cell cycle. The function $f_i^{(k)}(t)$ quantifies the gain or loss of k-clusters in subcompartment i of the cell cycle at a specific time t.

Specific restrictions on the model

Some assumptions are made about the model which restrict it to a more realistic situation in our experiments by limiting the number of unknown parameters to a minimum.

(a) *Time of observation:* the analysis will be restricted to an observation time of less than one cell cycle. Thus, all cells are allowed to divide only once. No statements are made about the later fate of the multiplets. Therefore T_{G_1} and T_C need not be estimated.

(b) *Cluster-creating function:* during the short time of observation, spatial rearrangement in the tissue can be neglected. New clusters can only be formed by cell division. Consequently clusters can only leave their pool at the end of the cell cycle (from the n-th subcompartment) and can only enter into the G_1 phase (first subcompartment). This assumption restricts $f_i^{(k)}$ to:

$$f_i^{(k)}(t) = 0 \text{ for all } i \geq 2. \tag{3}$$

The 'baseline' production of singles is taken into account in a very simplified way (recycling):

$$f_1^{(1)}(t) = C^{(1)} \cdot Y_n^{(1)}(t), \text{ where } C^{(1)} \text{ is defined in (4c).} \tag{4a}$$

The rationale is that most of the remaining singles belong, in fact, to recently produced pairs whose partner immediately left the plane of observation or lost their label. Thus a certain 'recycling' of singles seems to be justifiable. Other mechanisms are neglected.

A production of pairs due to spatial rearrangement of labelled cells or loss of label in triplets is assumed to be statistically very unlikely. Thus, only production due to division of labelled singles is assumed

$$f_i^{(2)}(t) = C^{(2)} \cdot Y_{n-1}^{(1)}(t), \text{ where } C^{(2)} \text{ is defined in (4d).} \tag{4b}$$

The values for $C^{(1)}$, $C^{(2)}$ determine the final plateaux of $Y^{(1)}$ and $Y^{(2)}$ after division and can be directly obtained from experimental data on FLC_1 and FLC_2:

$$C^{(1)} := \mathrm{FLC}_1 \text{ (plateau)}/\mathrm{FLC}_1 (t = 0) \tag{4c}$$

$$C^{(2)} := \mathrm{FLC}_2 \text{ (plateau)}/\mathrm{FLC}_1 (t = 0). \tag{4d}$$

(c) *Initial values:* all i subcompartments in a given pool contain the same number of cells and the labelling procedure labels all cells equally in S phase at $t = 0$. This determines the initial values of the pools. If all cells belonging to a k-cluster make up the fraction FLC_k of all labelled cells directly after labelling, all $n_S^{(k)}$ subcompartments of the S phase are attributed the same initial value

$$g^{(k)} := \mathrm{FLC}_k (t = 0)/n_S^{(k)}. \tag{4e}$$

(d) *Transit rates:* the coefficients λ_i are determined by the number of subcompartments and the duration of the phases of the cell cycle. They are identical within each cycle phase. This implies a gamma distribution of the cell cycle phases.

As a consequence of these special assumptions (*a*)–(*d*), the general system (1), (2) simplifies to the following formulae for the time development ($k = 1,2$):

$$\frac{d}{dt} Y_i^{(k)}(t) = \begin{cases} f_1^{(k)} - \lambda_1 \cdot Y_1^{(k)} & i = 1 \\ \lambda_{i-1}^{(k)} \cdot Y_{i-1}^{(k)} - \lambda_i^{(k)} \cdot Y_i^{(k)} & i = 2, \ldots, n^{(k)} \end{cases} \tag{5}$$

for the initial values after labelling:

$$Y_i^{(k)}(0) = \begin{cases} 0 & i = 1, \ldots, n_{G_1}^{(k)};\ G_1 \text{ phase unlabelled} \\ g^{(k)} & i = n_{G_1}^{(k)} + 1, \ldots, n_{G_1}^{(k)} + n_S^{(k)};\ S \text{ phase labelled} \\ 0 & i = n_{G_1}^{(k)} + n_S^{(k)} + 1, \ldots, n^{(k)};\ G_2 + M \text{ phase unlabelled} \end{cases} \tag{6}$$

and for the transition rate constants:

$$\lambda_i^{(k)} = \begin{cases} n_{G_1}^{(k)}/T_{G_1}^{(k)} & i = 1, \ldots, n_{G_1}^{(k)} \\ n_S{}^{(k)}/T_S{}^{(k)} & i = n_{G_1}^{(k)} + 1, \ldots, n_{G_1}^{(k)} + n_S{}^{(k)} \\ n_{G_2+M}^{(k)}/T_{G_2+M}^{(k)} & i = n_{G_1}^{(k)} + n_S{}^{(k)} + 1, \ldots, n^{(k)} \end{cases} \quad (7)$$

Parameter estimation

This model of cluster production can now be used to extract information on cell phase durations from experimental data.

The parameter $g^{(1)}$, $g^{(2)}$, $C^{(1)}$ and $C^{(2)}$ can be experimentally determined from the initial and plateau values of FLC_1 and FLC_2 data. In fact, we chose the average values of the first two or three data points for FLC_1 ($t = 0$) and the average of the plateau values for FLC_2 (plateau) and FLC_1 (plateau). According to eqns (4c), (4d) and (4e), this gives $C^{(1)}$, $C^{(2)}$ and $g^{(1)}$, $g^{(2)}$. Then four parameters remain to be estimated from the data for each type of cluster: cell phase durations: T_S, T_{G_2+M} and number of subcompartments: n_S, n_{G_2+M}. Instead of n_S and n_{G_2+M} it is convenient to use the variances of the phase durations:

$$V_S = T_S{}^2/n_S, \quad V_{G_2+M} = T_{G_2+M}^2/n_{G_2+M}. \quad (8)$$

For any given set of values (T_S, T_{G_2+M}, V_S, V_{G_2+M}) theoretical curves can be integrated for singles and pairs. We use an integration interval of 0·01 hours. In order to quantify their fit to the data we employed as an error function the least square value (error value = square root (sum of squared differences between the model curve and data points/number of data points)) between theoretical and experimental values. In principle, we have to screen a four-dimensional parameter space to find the set of parameters which generates a minimum error value (ideally zero). In practice we could considerably reduce the numerical effort by the following procedure:

(1) Rough estimate of T_S, T_{G_2+M}: only integer values between 1 and 12 hr were considered. For given fixed variances ($V_S = V_{G_2+M} = 0{\cdot}1$ or 0·2, 0·5, 1·0) we could select those T_S, T_{G_2+M} values amongst the 144 combinations with small error values.

(2) Refined estimate of T_S, T_{G_2+M}: for each of these pre-selected T_S, T_{G_2+M} candidate values (derived from (1)) we could select those V_S, V_{G_2+M} values amongst the 10 × 10 combinations of variances (0·1 increments from 0·1 to 1·0) with the smallest error value. Thus, for each of the pre-selected T_S and T_{G_2+M} values, one obtains the best V_S, V_{G_2+M} combination.

(3) Acceptable deviation from the best fit case: the search procedure for best V_S, V_{G_2+M} values was repeated for many different combinations of integer values of T_S, T_{G_2+M} to obtain the overall best fit combination with the lowest error value. Then all T_S, T_{G_2+M} combinations were accepted as reasonable alternatives if their best error value (by V_S, V_{G_2+M} selection) was within 10% of the overall optimum. This gave rise to ranges of acceptable T_S, T_{G_2+M} estimates, which generally differed slightly for the single and pair data. In order to take advantage of the two separate estimates from singles and pairs, only overlapping ranges of acceptable 10% estimates were accepted for the final conclusion. In other words we accepted only those cases where both estimates from the singles and pairs are simultaneously within 10% of their respective minimum values (Fig. 6 summarizes the range of acceptable values within 10% of the overall best fit values, as well as the corresponding 5% situation).

RESULTS

Data from dorsal skin of mice

In Figs. 2 and 3 the singles and pairs from actual labelling experiments using sheets of the basal layer of the dorsal skin of mice have been analysed. The initial LI values for the 03.00 hours and 15.00 hours labelling experiments were 6·0% and 2·3%, respectively. The initial values for the fraction of singles were 0·67 and 0·88. The initial values for the fraction of pairs (FLC_2 (0)) were 0·23 and 0·125, respectively (higher clusters were neglected). These values are consistent with the random distribution of labelled cells based on the assumption that each cell has six neighbours. The computer-derived curves show a good agreement with the data points. The phase duration estimates are presented in Table 1.

In a second 15.00 hours experiment, more frequent measurements were performed (Fig. 4). The data show more scattering than the comparable experiment in Fig. 3 and consequently it is obvious that the computer-aided parameter estimation will be less accurate. Table 1 shows, indeed, that the range of acceptable T_S and T_{G_2} values is broader, but covers a very similar area.

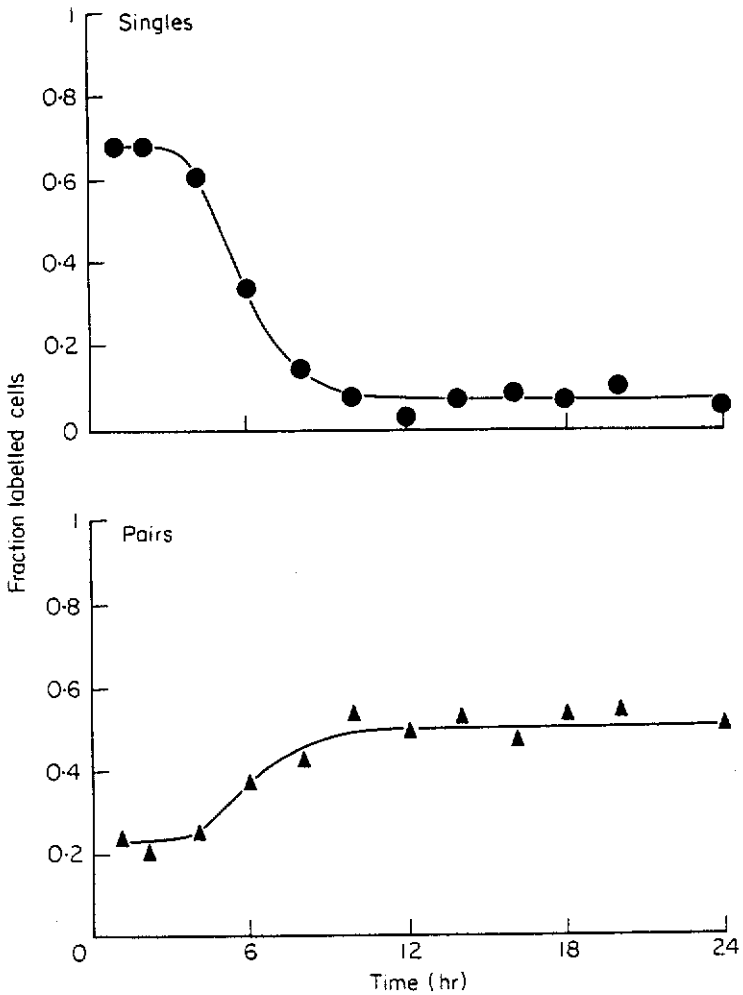

Fig. 2. Data are shown for the behaviour of singles (●) and pairs (▲) in sheets of the basal layer of mouse epidermis labelled at 03.00 hours (experiment 1). The best computer-fitted curves (——) are shown for the singles and pairs (best combinations in Table 1).

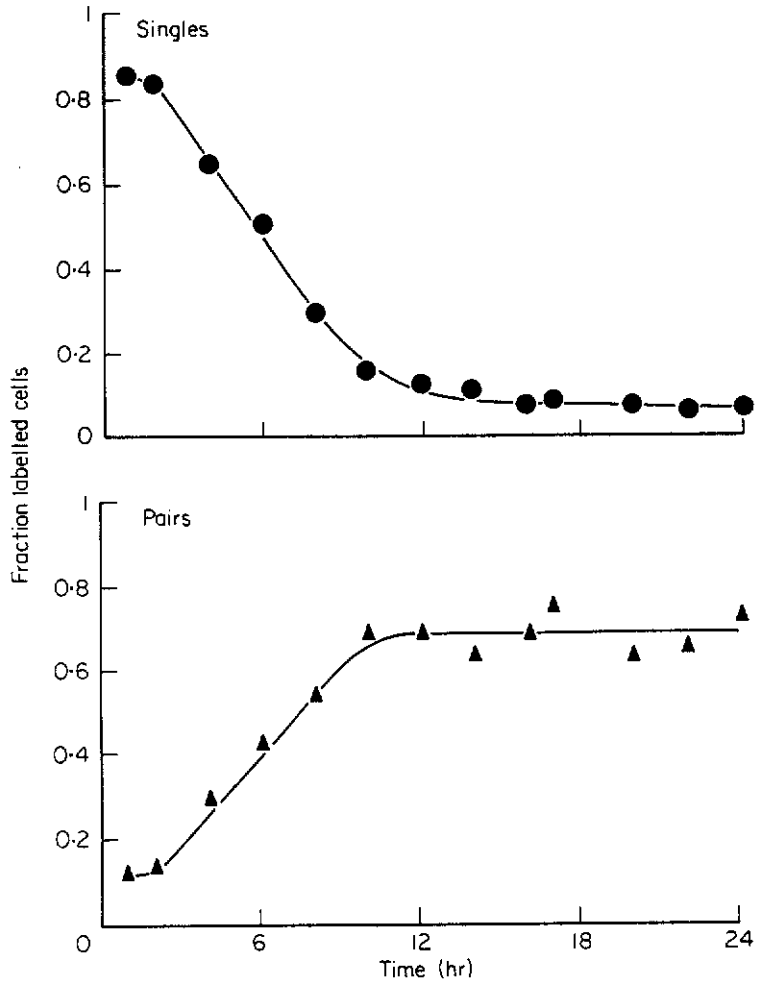

Fig. 3. Sheets of basal layer of mouse epidermis labelled at 15.00 hours (experiment 2) (see legend of Fig. 2 and Table 1).

In a third 15.00 hours experiment measurements were made from *sections* (Fig. 5). Most notably here, the final plateaux of singles and pairs stay closer to the initial values than in the previous cases. Apparently a large number of singles divide so that the daughters remain in the basal layer but out of the plane of the section. Thus, many of the new pairs are not seen, which keeps the singles at relatively high values. This effect, together with the large experimental scatter, makes the parameter estimation even more difficult than in Fig. 4. Table 1 exhibits the large range of acceptable values. Apparently these data suggest a longer T_{G_2+M} than the other two 15.00 hours experiments.

In all preceeding figures theoretical curves are shown, which generate the best fit to the data. Their parameters are given in Table 1. Singles and pairs yield almost identical results.

Figure 6 gives a more detailed presentation of the 15.00 hours experiments showing the domains of acceptable T_{G_2} and T_S values for the three different experiments discussed. A combination value is acceptable if the associated minimum error value is not more than 10% larger than the error value of the best fit. In Fig. 6 domains are only shown when both single and pair data simultaneously provide acceptable minimum error values within the 10% (and 5%) margin.

Table 1. Cell cycle phase durations derived from the cluster analysis

Mouse epidermis (basal layer)	T_S	V_S	T_{G_2+M}	V_{G_2+M}	$T_S + T_{G_2+M}$	Error value*	Figure
(1) Experiment (sheets) (Labelling at 3.00 hr)							
Singles (best fit)	3	0·5	4	0·2	7	0·0167	
Pairs (best fit)	6	0·1	3	0·1	9	0·0234	
Best combination (single, pair)	3	0·5, 0·6	4	0·2, 0·2	7	0·0167, 0·0245	Fig. 2
(2) Experiment (sheets) (Labelling at 15.00 hr)							
Singles (best fit)	8	0·3	2	0·3	10	0·0137	
Pairs (best fit)	7	0·2	2	0·5	9	0·0327	
Best combination (single, pair)	8	0·3, 0·1	2	0·3, 0·2	10	0·0137, 0·0335	Fig. 3
(3) Experiment (sheets) (Labelling at 15.00 hr)							
Singles (best fit)	10	0·1	2	0·1	12	0·0607	
Pairs (best fits)	10	0·1	2	0·1	12	0·0383	
	7	0·1	4	0·5	11	0·0383	
Best combination (single, pair)	10	0·1, 0·1	2	0·1, 0·1	12	0·0607, 0·0607	Fig. 4
(4) Experiment (sections) (Labelling at 15.00 hr)							
Singles (best fits)	4	0·1	4	0·6	8	0·0443	
	7	0·1	3	0·4	10	0·0443	
Pairs (best fits)	4	0·2	4	0·6	8	0·0456	
	7	0·1	3	0·4	10	0·0456	
Best combination (single, pair)	7	0·1, 0·1	3	0·4, 0·4	10	0·0446, 0·0460	Fig. 5

* Error value is the square root of the sum of the squared differences between the data points and the model curve over the number of data points.

DISCUSSION

A new method is proposed which involves an analysis of the clustering of labelled cells. It permits information to be extracted on the S and the G_2 + M phase duration and their variances. Besides that, the following statements can be made. The technique can be applied easily during the early phase of a labelling experiment, i.e. between the time of labelling and before the second mitosis of the labelled cells. Unlabelled cells need not be counted. The technique can be applied to all levels of clustering, but for practical reasons may be restricted to the analysis of labelled singles and pairs. It is not necessary to have the whole spatial information about the neighbourhood of cells. For example, it is possible to consider only the clusters in a two-dimensional section although labelled cells may also occur in the third dimension, since some cell divisions and migrations take place in the plane of the section. This method could therefore be applied, for example, to solid tumours or regenerating liver where the plane of section relative to the three dimensions of the tissue may not be known.

Using the changes in the proportion of singles and pairs for the estimates for T_S and T_{G_2+M}, slightly different values are obtained. The influences of [^{3}H]TdR (see Wichmann *et al.*, 1985) or the effects of the circadian rhythm (see Wichmann & Fesser, 1982) on the changes in clustering have been neglected. However, factors which influence the absolute number of labelled cells would be expected to have relatively little effect, because we only compare relative changes of one labelled cohort versus another. The effects of tissue and kinetic heterogeneity, [^{3}H]TdR and circadian rhythms will be discussed in more detail elsewhere. In

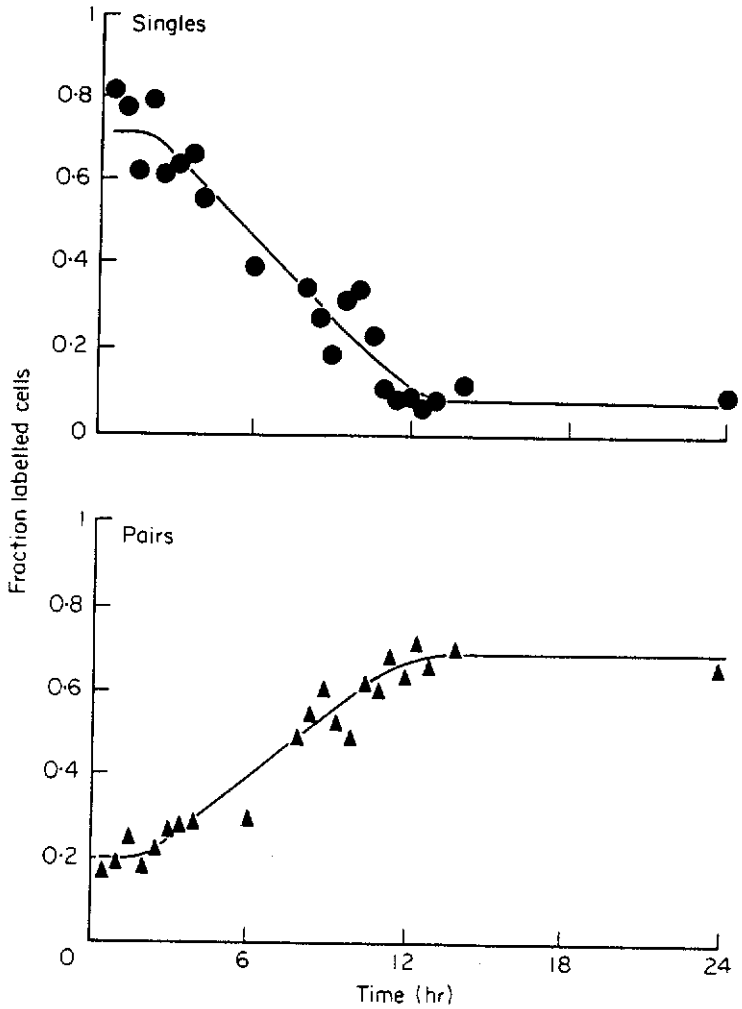

Fig. 4. Sheets of basal layer of mouse epidermis labelled at 15.00 hours (experiment 3) (see legend of Fig. 2 and Table 1).

tissues with a pronounced circadian rhythm, both the PLM technique and the technique presented here may run into difficulties unless the full implications of the rhythms are taken into account in the method of analysis (Clausen *et al.*, 1979; 1981, Wichmann & Fesser, 1982). The technique described here has the following advantages in comparison with the PLM technique. It is technically very simple to perform. It is largely independent of the factors which influence the absolute number of labelled cells. It provides two simultaneous, but independent, estimates for the duration of the S and G_2 + M phases (singles and pairs). The technique allows good estimates of T_S, T_{G_2+M}, V_S and V_{G_2+M} if (1) the system under investigation has a low initial LI (many initial singles and few pairs); (2) the cell cycle time T_C is long compared with $T_S + T_{G_2+M}$ (which provides a well-defined final plateau); (3) T_S is roughly of the same order as T_{G_2+M}, which results in relatively sharp edges; (4) the plane of cell division coincides with the plane of observation, such that the disappearance of singles and reappearance of pairs can be comprehensively recorded (i.e. the initial and final plateaux differ maximally); (5) the experimental scatter is small and there are many data points in the changing parts of the curves (S phase); (6) migratory activity within the basal layer can be neglected (i.e. there are stable pleateaux); (7) the biological variances of the cell phase durations are small, resulting in sharp edges. However, this is largely an unknown parameter.

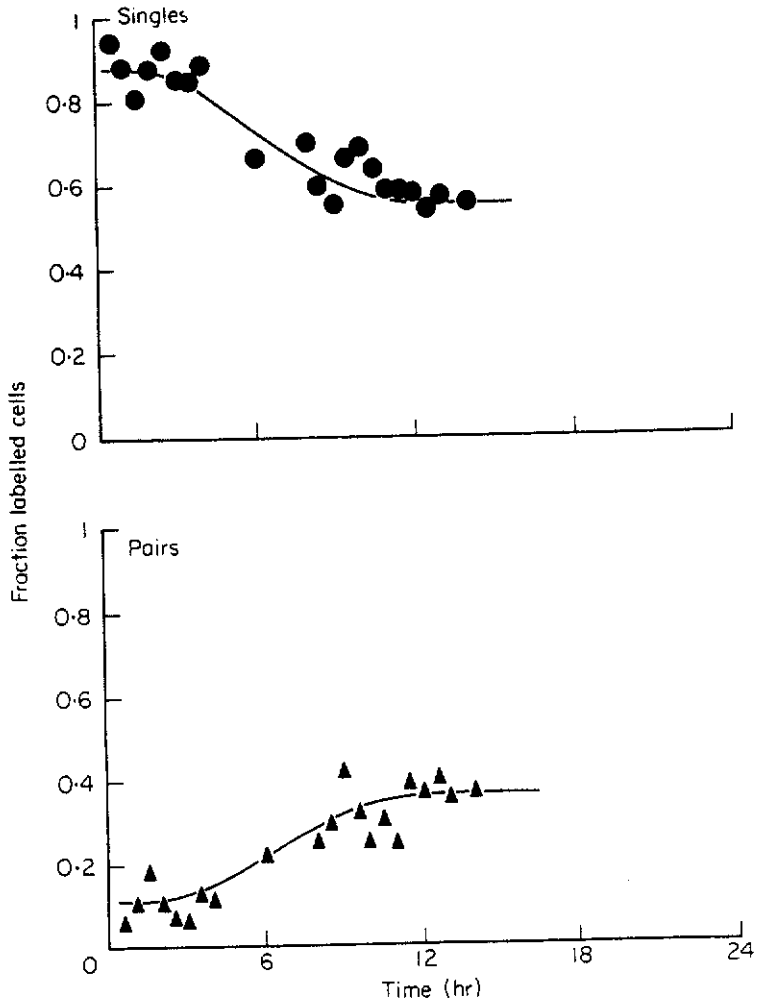

Fig. 5. Sections of basal layer of mouse epidermis labelled at 15.00 hours (experiment 4) (see legend of Fig. 2 and Table 1).

Apparently, these conditions were largely fulfilled in Figs. 2 and 3. Condition (5) was not fulfilled in the more detailed 15.00 hours experiment (Fig. 4) which resulted in a less accurate (but still acceptable) parameter estimation. Conditions (4) and (5) are largely violated in the section experiment (Fig. 5), where the estimates of T_S and T_{G_2+M} almost fail. However, the estimate for the sum $T_S + T_{G_2+M}$ is still acceptable. Clearly, in many tissues and tumours some of these requirements may not be met while in others they may be; in the latter case the technique may be of value. The greatest precision on the values will be achieved if many (greater than 10) data points are obtained over a time scale estimated to exceed the time of $T_S + T_{G_2+M}$. It can be argued that the sum of $T_S + T_{G_2+M}$ is a very robust parameter, which can still be estimated even when the separate estimates for T_S and T_{G_2+M} fail. Indeed, Fig. 6 shows that all the 15.00 hours estimates have very similar $T_S + T_{G_2+M}$ values.

The variances V_S, V_{G_2+M} turn out to be rather insensitive parameters. In most good fit cases they range between 0·1 and 0·5, which we classify as small to medium. It should be noted that larger variances generally produce bad fits, indicating that neither S nor G_2 phases obey a pure random transition characteristic ($V = 1{\cdot}0$). The differences between the 03.00 hours and 15.00 hours experiments can be explained by circadian rhythms (Tvermyr, 1972, Clausen *et al.*, 1979), which are also known to be present in the skin of our mice (Potten *et al.*, 1977).

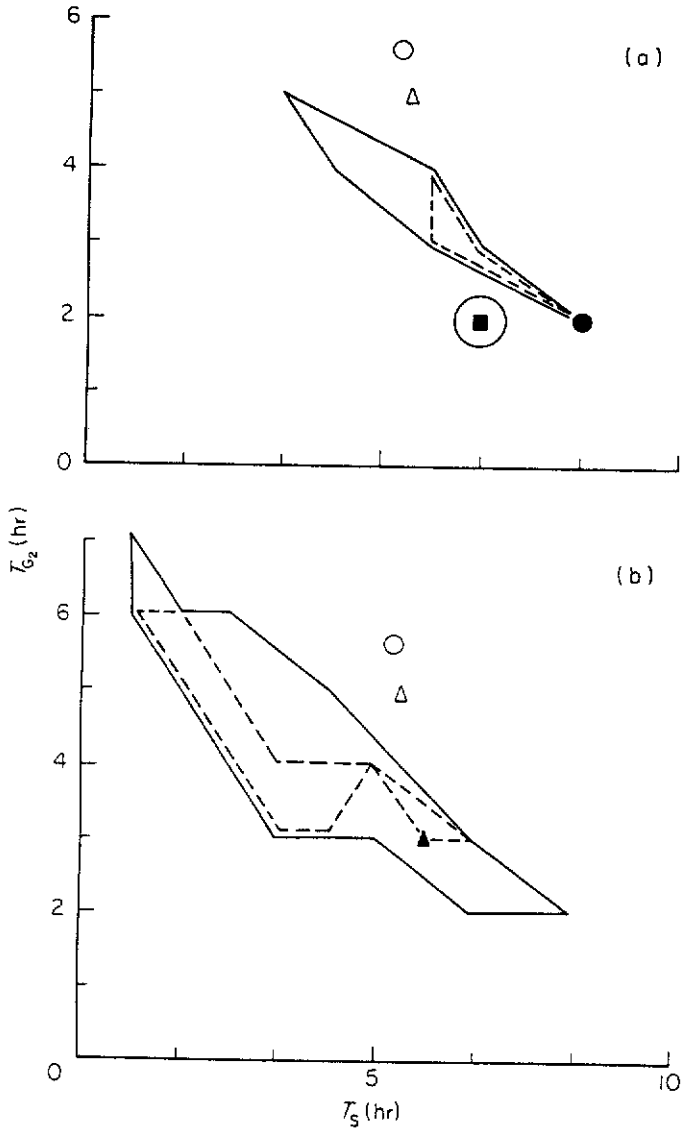

Fig. 6. The contour lines indicate the 10% (——) and 5% (– – –) deviations from the best fit of single and pair combinations.

The three 15.00 hours experiments are presented as follows.

Fig. 6a: experiment 2: best fit, ■

experiment 3: best fit, ●

Fig. 6b: experiment 4: best fit. ▲.

The open symbols indicate PLM-derived estimates for T_S and T_{G2+M} from 15.00 hours experiments: ○ (Potten *et al.*, 1985); △: (Wichmann *et al.*, 1985).

The differences between the two 15.00 hours sheet experiments are small, and can be attributed to different experimental variability. The large range in acceptable values for the 15.00 hours section data suggests that these estimates should not be taken too seriously. However, the sum of $T_S + T_{G_2+M}$ is similar to that in the other two 15.00 hours experiments (Fig. 6). It should be noted that $T_S = 8$ hr and $T_{G_2} = 2$ hr is a set of parameters that can fit most of the 15.00 hours experiments discussed.

A comparison of our results with PLM derived values is also given in Fig. 6. These PLM data (open symbols) were, however, obtained in different experiments using DBA2 mice instead of C57B1 × DBA2 F1 (=BDF_1) cross used here. The reasons for the discrepancies

are unknown and could be studied if a simultaneous experiment of PLM and labelled clusters was attempted. This new method could not replace the PLM analysis, but it would, under certain circumstances, provide additional information where the PLM might give erroneous underestimates (e.g. very short S phase or few data points) or overestimations (^{3}H|TdR re-utilisation) or is otherwise difficult to obtain. The present method is, however, simpler than the PLM, is less labour-intensive and is statistically more reliable than the LI method.

In summary, a new method is proposed for obtaining phase durations for S and G_2 + M. The information used is the spatial arrangement of labelled cells in epidermis obtained at various times after labelling. Such data have been subjected to a rigorous mathematical analysis and curve fitting procedure.

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