LABELLING OF DNA AND CELL DIVISION IN SO CALLED NON-DIVIDING TISSUES

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ABSTRACT

Autoradiographs of adult mice killed at various times after injection of tritiated thymidine show significant numbers of labelled nuclei in organs in which mitoses are either very rare or completely absent. The proportion of labelled cells that divide was estimated from the decline in the number of grains per nucleus, the number of pairs of labelled cells in sheets of epithelium in squashes, the number of labelled metaphases after 6 hours' treatment with Colcemid, and the ratio of mitotic index to labelling index. The longest possible duration of G_2 in the epithelial cells of seminal vesicles was deduced from the results of Feulgen photometry. The results show that only a small proportion of the labelled cells divide in the seminal vesicles and liver, whilst probably none divide in brain, smooth muscle, and heart muscle. It is suggested that, in the so called "non-dividing tissues" of adult mice, cells periodically renew their DNA by a process the details of which are as yet unknown.

Various attempts to correlate cell division and the number of cells incorporating tritiated thymidine and other precursors into DNA in adult animals have led to the conclusion that in some organs, such as intestines and esophagus, all labelled cells divide, whilst in others more cells become labelled than eventually divide (8, 10). For the latter organs, important parameters such as duration of DNA synthesis and of mitosis are not known with sufficient accuracy to make the results of any one experiment conclusive. New experimental approaches are described in this paper and the results obtained are correlated with those of previous experiments by the present author and with the results of biochemical investigations showing considerable instability of DNA in liver, muscle and kidney (2, 4, 5).

In the first group of experiments using adult mice, described here, tritiated thymidine was used to label DNA, and Colcemid to arrest cells in mitosis. Fixations were made in a time sequence which permitted the determination in various organs of the total number of labelled cells which attempt division during the experiment. It will be shown that this result is not influenced by diurnal rhythms or by variations of either the labelling index or the duration of the various periods in the mitotic cycle. It is only assumed that those cells of the labelled cells that divide do so according to a reasonably constant time-table, and that the proportion of all labelled cells that divide is the same in different animals.

The second group of experiments depends on the fact that when a labelled epithelial cell divides the two labelled daughter cells will be found side by side and are likely to remain so for some time. Sheets of epithelium were found in squashes prepared by the method described by Gall and Johnson (3), and the ratio of pairs of labelled cells to singles, which indicates the proportion of labelled cells that have divided (the dividing index), was determined. Another measure of this index was calculated from the difference in the average number of grains per nucleus at various times after injection of tritiated thymidine.

For want of a better name, the term "dividing tissues" is used for tissues such as intestinal epithelium or those of esophagus where mitoses are

frequently seen, and the term "non-dividing tissues" for those where mitoses are completely absent or very scarce, for example, brain tissue and tissues of the liver and seminal vesicle.

MATERIALS AND METHODS

EXPERIMENT 1: Twenty adult mice of an inbred strain were given intraperitoneal injections of 27 μc of tritiated thymidine. Four animals were killed after one hour. At that time two animals were injected with 25 μg of Colcemid, a derivative of colchicine (CIBA Ltd., Basel, Switzerland), and killed 6 hours later. Additional animals were treated with Colcemid and killed as shown in Table I. Organs were fixed for 1 hour in acetic acid–alcohol (1:3) and

TABLE I

Plan of Colcemid Experiment

All animals were injected with H^3-thymidine at time O.

Number of animals (mice)	Colcemid injected at	Killed at
	Hours after injection of H^3-thymidine	
4	—	1
2	1	7
2	7	13
2	13	19
2	19	25
2	49	55
2	73	79

for 24 hours in formol-saline and embedded in paraffin. Sections were stained by the Feulgen reaction before applying AR-10 stripping film.

EXPERIMENT II: Seminal vesicles of the animals killed 1, 6, 12, and 24 hours after injection of tritiated thymidine were stained in bulk by the Feulgen reaction and squashes were prepared as described by Gall and Johnson (3).

RESULTS

Cell Counts

All, or most, cells which have started mitosis during 6 hours' treatment with Colcemid will be arrested in metaphase. Those which synthesized DNA during the time tritiated thymidine was available will be labelled, and the proportion of labelled divisions as a percentage of all labelled cells can be determined by counts. Similar counts during later periods will have to be corrected to take into account the increased number of labelled cells. If out of 100 labelled cells the number d_1 divide during the first period of Colcemid treatment, the number of labelled cells at the end of that period will be $100 + d_1$. If, at the end of the second period, out of 100 cells the number d_2 are found to be in division, the true rate of division, d'_2, per 100 of the cells that were originally labelled will be

$$d'_2 = d_2\,(100 + d_1)/100 \qquad (1)$$

and similarly for subsequent periods of Colcemid treatment.

The results of our counts, presented in Table II, show that only 5.8 to 12.2 per cent of the labelled nuclei divide in seminal vesicle, smooth muscle, and heart muscle, as compared with 79 per cent for esophagus and jejunum. It must be emphasised that in view of the low number of labelled cells and divisions the accuracy of the count is not so high as might be desirable. Repeat counts indicate that the variability of the cumulative dividing indices may be as high as 5 per cent.

Two figures, 12.2 per cent and 15.1 per cent, are given for the seminal vesicles. The lesser figure is derived from the three shorter times only, omitting the values for 49 + 6 hours and 73 + 6 hours which, for the following reasons, can be regarded as due to second divisions. The labelling index in this experiment was 0.8 per cent at 1 hour after injection of tritiated thymidine. Incorporation into DNA takes 6 to 8 hours (9) as in "dividing tissues," and the percentage of cells incorporating each day would be 2.4 to 3.2 per cent. Therefore, the average interval between subsequent periods of synthesis would be 31 to 42 days, say 35 days, for a cell. If the postsynthetic period (G2) lasted for 1 day, one out of 35 nuclei, nearly 3 per cent, should contain 4C amounts of DNA. Richards (11) failed to find by Feulgen-photometry any such cells out of 250 interphase cells. Similarly, Gall and Johnson (3) did not find any amongst 100 unlabelled interphase nuclei. It, therefore, has to be assumed that the period G2 in epithelial cells of the seminal vesicle cannot last longer than 24 hours since a total of 10 cells (3 per cent) with 4C values should have been found amongst 350 cells.

Table II also shows that the cumulative mitotic index is 79 per cent in esophagus and jejunum. It is not clear whether or not the remaining 21 per cent of mitoses which were not scored is significant. It is known that the yield of C mitoses declines when treatment with colchicine lasts

longer than 4 hours. If this were the case, a similar decline would have to be assumed for other organs, and the values for the cumulative mitotic indices corrected accordingly. Other possibilities to explain this discrepancy are being explored.

Grain Counts

If all labelled cells in an organ divide, the average number of grains per nucleus should be halved. If we find that after 1 hour's treatment with H^3-thymidine 100 nuclei show an average of n_1 grains and that the number d of them have divided after time t, $100 + d$ nuclei should still show $100\ n_1$ grains. If the average number of grains per nucleus at that later time is n_t, the following equations can be derived.

$$100\ n_1 = n_t(100 + d) \tag{2}$$

$$d = 100\ \frac{n_1 - n_t}{n_t} \tag{3}$$

Grain counts on autoradiographs of sections of seminal vesicles gave an average of 15.7 ± 1.4 grains per nucleus at 1 hour after injection and 14 ± 1.2 grains for the pooled counts for the longer times. The difference between two grain counts governs Equation 3 and, therefore, this estimate is not accurate but certainly shows that only a small proportion, approximately 12 per cent, of the cells can have gone through mitosis. The possibility that all cells might have divided can be excluded, since a grain count of half the value at 1 hour, namely 7.8, would have to be obtained instead of 14 ± 1.2.

Pairs of Labelled Cells

Gall and Johnson (3), whose paper will be referred to in more detail in the Discussion, based recognition of nuclei, for their analysis, on the assumption that all epithelial nuclei in the seminal vesicle appear to be circular in squashes. Repeating their experiment, we realised that this assumption is not justified. "Sheets" of tissues are seen in the squashes, and elliptical nuclei are frequently found together with round ones in remnants of epithelial as well as connective tissue. Similar observations were made on sections where it was also noticed that cells in division frequently leave the orderly linear arrangement of nuclei. Only few epithelial cells that are still in such a sheet are sufficiently well separated from their neighbours to be suitable for Feulgen-photometry. It was concluded that, for Feulgen-photometry and grain-counts, single cells that are well separated from adjacent cells on a squash are subject to uncertainties of recognition and selection whilst whole sheets are unsuitable for the reasons mentioned above.

The sheets of epithelium were, however, found to be very suitable for scoring the dividing index. During division, a labelled cell will produce two labelled cells which are very likely to remain close together for some time. Therefore, counts of single labelled nuclei and pairs of labelled nuclei can show the proportion of labelled cells that has

TABLE II

Cumulative Mitotic Indices in Various Organs of the Mouse

Treatment	Seminal vesicle	Smooth muscle	Heart	Jejunum	Esophagus
	Per cent labelled mitosis of labelled cells				
1 + 6	5.6	0	1.8	15.4	23.2
7 + 6	3.5	0	0	18.5	13.1
13 + 6	2.7	4.5	2.6	15.2	12.5
19 + 6	0	2.6	1.3	12.8	12.8
49 + 6	1.3	0	0	—	1.5
73 + 6	1.2	0	0	—	0
Cumulative mitotic index	12.2 (15.1)	7.1	5.8	79.3	79.4

Treatment: the first figure denotes the time in hours after injection of H^3-thymidine at which Colcemid was injected, the second the duration of treatment with Colcemid.

divided. Table III shows that at 1 hour after injection 12 ± 3.5 per cent of all labelled cells were pairs (counting a pair as one), and that at later times this figure increased to 25 ± 4.1 per cent. Since no labelled cells will have divided at 1 hour after injection, pairs found at that time must be the result of adjacent cells' having synthesised DNA at the same time. This view is supported by the observation that very occasionally triplets or double pairs of labelled cells are seen at later times, also showing that the progeny of a division remains in close proximity for a considerable length of time. The dividing index will, therefore, be the proportion of labelled pairs at that time minus the proportion at 1 hour. It can be concluded that 13 per cent of the labelled epithelial cells have divided.

The average number of grains for both nuclei of a pair (12 hours after injection) was found to be 101 grains, for a few pairs 56 ± 4 grains. It is reasonable to assume that the "heavy" pairs are made up of nuclei which incorporated H^3-thymidine and have not divided, whilst the others represent daughter cells of a division.

TABLE III

Labelled Cell Pairs in Sheets of Epithelial Cells in Squashes of Seminal Vesicles

Time after injection (hours)	1	6	12	24
Per cent pairs	12 ± 3.5	19 ± 4.7	15 ± 4	25 ± 4.1

DISCUSSION

Incorporation of labelled precursors into DNA at the same rate as that observed in tissues known to be dividing might represent premitotic synthesis or increase of the DNA content to 4C or 8C values without subsequent division. Whether or not DNA synthesis, as indicated by incorporation of H^3-thymidine, leads to division can be ascertained (*a*) by a correlation of the number of mitoses with the number of labelled cells, (*b*) by counts of the number of positive nuclei shortly after labelling and at later times when most or all of the labelled nuclei should have divided at least once, (*c*) by scoring pairs of labelled cells, (*d*) by grain counts, and (*e*) by an estimation of the rate of migration or disappearance of cells in renewing tissues. Incorporation that has led to the formation of cells permanently containing 4C or 8C amounts of DNA can be detected by means of Feulgen-photometry or by biochemical determination combined with cell counts.

If incorporation leads neither to cell division nor to polyploidy, renewal of DNA or metabolic activity in the wider sense of the word can be assumed, unless DNA be considered chemically labile in such a way that exchange of bases is frequent. It seems reasonable to assume that chemical instability of DNA would show up in all or nearly all cells; if this instability is confined to a small fraction of the nuclei it would still indicate a different metabolic state in this proportion of cells.

The results obtained in the Colcemid experiment reported here indicate strongly that incorporation of labelled thymidine exceeds the requirements for cell division by a factor of approximately 8 to 12 in seminal vesicles, heart muscle, and smooth muscle; comparison of this figure with the dividing index in esophagus and small intestine shows that a significant difference exists between the known "dividing tissues" and the "non-dividing" ones. From data published by Messier and Leblond (7), a similar difference has been demonstrated (8). In intestinal and keratinising epithelia the average number of labelled cells is doubled within reasonable times after injection, whilst in non-dividing tissues it does not increase significantly.

Satisfactory agreement in the estimates of the proportion of the non-dividing labelled cells in the seminal vesicle has been reached by the different methods reported here. Though any one of these methods may be open to objections, the agreement of the numerical results can only be due to a common phenomenon. The assumptions made tacitly are discussed below. It can be concluded that these methods are largely independent of each other (Table IV).

The values under the heading of 10 *MI/LI* have been calculated by assuming that for dividing tissues the labelling index is approximately ten times the mitotic index, as found by Schultze and Oehlert (12) and by Messier and Leblond (7). Therefore, if *MI* is the mitotic index without

colchicine, the labelling index necessary for divisions should be 10 *MI*. If a labelling index *LI* is determined, the dividing index (*d*) will be:

$$d = \frac{10\ MI}{LI} \times 100 \qquad (4)$$

The following assumptions are inherent in the different approaches. For the combined use of colchicine and tritiated thymidine, it is assumed (*a*) that the dividing index is the same for different animals, (*b*) that the variability of G2 (the premitotic gap) is the same for a given organ for different animals within one experiment, and (*c*) that all labelled cells which are going to divide do so within the period of the experiment. Using grain counts for the estimate, the assumptions are (*d*) that there is no significant loss or gain of tritium from the labelled DNA during the time of the experiment, (*e*) that a labelled nucleus gives rise to two labelled ones after division, and assumptions (*a*) and (*e*). When pairs are counted, the assumptions are (*f*) that in the epithelium labelled daughter nuclei of a division remain close together, (*g*) that daughter nuclei survive intact and in the epithelium during the time of the experiment, and assumptions (*c*) and (*e*). In the comparison of labelling and mitotic indices it is assumed that (*h*) the ratio of the duration of DNA synthesis to that of mitosis is the same for different animals, (*i*) that either no labelled cells have as yet divided when the counts were made, or otherwise that assumption (*c*) is valid, and (*k*) that the variability of the labelling index between animals is within reasonable limits.

Assumptions *b*, *d*, *e*, *g*, *h*, and *i* are identical with those made in many studies on cell proliferation using labelled DNA precursors and autoradiography. Assumption (*c*) implies that not all labelled cells may divide; that it is justified is shown by the consistency of the results obtained in our experiments. Assumption (*a*) has also been discussed for the seminal vesicle in the section on Results. Feulgen-photometry has shown that G2 in this organ cannot last longer than 24 hours. No data are available for other organs. In the Colcemid experiment we did not find any labelled divisions in smooth muscle or heart muscle after 49 hours, 73 hours, or 7 days. The variability of the labelling index is too high to expect accurate results from comparing this with the very low mitotic index (assumption *k*). In none of our experiments, however, has the labelling index been sufficiently low to assume that in seminal vesicles or muscle all labelled cells proceed to division.

An experiment with multiple injections of labelled thymidine (8) showed that the phase during which cells incorporate thymidine lasts for 6 to 8 hours in the seminal vesicle, the same value as that frequently found in dividing tissues. The labelled cells showing Feulgen values between 2C and 4C found by Gall and Johnson (3) at 6 hours after injection can not be explained on the assumption of premitotic DNA synthesis. Practically all of the cells synthesising DNA at the time of injection and during the following short time (½ hour to 1 hour) of availability of H^3-thymidine should have finished DNA synthesis when the mouse was killed.

TABLE IV

Proportion of Labelled Cells Dividing in Mouse Seminal Vesicle

	Method			
	Colcemid	Grain counts	Pairs	10 MI/LI
Proportion	0.12	0.12	0.13	0.03–0.23
Assumptions (see text)	*a*, *b*, *c*	*c*, *d*, *e*	*c*, *f*, *g*	*h*, *i* or *c*, *k*

It can be seen (Table IV) that, with the exception of assumption (*c*), the four methods used are independent of each other. These results agree as well as can be expected, and it can be concluded that in the seminal vesicles not more than 12 per cent of the labelled cells proceed to division.

Gall and Johnson (3) prepared autoradiographs of Feulgen-stained squashes of seminal vesicles of mice killed 6 hours after injection of H^3-thymidine. The labelling indices were determined and one slide which had a high labelling index was selected for further work. After counting of grains and determining the labelling index the photographic image was removed and the Feulgen absorption of 100 previously labelled and 100 unlabelled nuclei was measured. These authors found that of

the labelled nuclei 37 had 2C values of which 15 were seen as pairs; approximately ½ of the remainder were between 2C and 4C, the other half 4C, resulting in a linear correlation between grain number and Feulgen dye content. Two labelled cells were in metaphase and 4 in telophase. In evaluating the significance of their findings it should be borne in mind that only one slide was investigated. Even if all 37 labelled 2C cells were daughter cells of divisions, the other 63 might or might not divide; that there is a correlation between Feulgen content and grain number for the remaining nuclei neither proves nor disproves premitotic DNA synthesis. The authors themselves admit that 6 labelled divisions are insufficient as the basis for a reliable mitotic index.

Some of the points raised by Gall and Johnson (3) have been cleared up by our findings. The results of the Colcemid experiment and the counts of pairs in sheets show that a diurnal rhythm cannot be responsible for the excess of labelling over the mitotic index; the determination of the proportion of pairs of labelled cells over singles shows that not all labelled cells divide, if the experimental conditions are the same as in Gall and Johnson's experiments.

It is difficult to assess the significance of Gall and Johnson's work. Out of the large number of slides examined we found one showing approximately three times the number of divisions that should have been expected from the average mitotic index, though the sample was too small to be statistically significant. The squashes derived from one animal killed 12 hours after injection showed 40 per cent pairs. It is possible that by chance there was a higher rate of mitosis on the slide investigated by Gall and Johnson. Otherwise, if of more general validity, their findings would suggest that premitotic and non-mitotic DNA syntheses have some feature in common, which after all is not so surprising.

In smooth muscle or heart muscle no dividing cells could be definitely identified. All cells which looked remotely as though in metaphase were accepted in our counts, the results of which should, therefore, be regarded as upper limits. It is not known whether polypoid nuclei are present in muscle, though biochemical analyses make it unlikely that the incidence is high, and an unusually long G2 can be excluded because the average amount of DNA per cell would have to be higher than 2C if a considerable number of cells remained in G2.

A number of papers dealing with the incorporation and subsequent loss of tritiated thymidine have been published recently. Gerber *et al.* (5) injected adult rats with H^3-thymidine, killed the animals at various times after injection, and determined the specific activity of the DNA of various organs. In small intestines, spleen, and thymus the specific activity at first declined rapidly at a rate equivalent to a biological half-life of 1.2, 3, and 1.8 days respectively, then at a slower rate equivalent to 21, 28, and 49 days. For the non-dividing tissues the biological half-lives were 14 days for liver, 18 days for muscle, and 39 days for kidney. The amounts of thymidine incorporated in these organs were high, the specific activity of the DNA of the small intestine being only 20 to 26 times higher than in liver. It is, of course, well known that it is much easier to find 20 mitoses in sections of the small intestine than to find one in liver (see Table V).

Garder and Devik (4) injected adult mice with H^3-thymidine and determined the activity per milligram of tissue at various times after injection. Separate determinations showed 76 to 92 per cent of the activity in DNA. Analysis of their published results shows in all organs a relatively rapid fall in activity with a biological half-life of 2.8 to 7 days followed by a slower fall at rates of approximately 20 to 35 days. At short times after injection, the difference between specific activities in non-dividing and dividing organs was considerably less than would be expected from the rates of cell division. Devik and Halvorsen (2) found considerable stability of H^3-thymidine incorporated in mouse liver after hepatectomy, but a loss of 48 per cent after 30 days and 74 per cent after 118 days in controls.

Chang and Vetrovs (1) recovered considerable amounts of C^{14} when cultures of human amnion cells labelled with C^{14}-thymidine were transferred to unlabelled medium. Mitotic counts and cell counts make it unlikely that a balance of cell death and division accounts for their findings.

Our own findings (8), based on counts of labelled cells, suggest biological half-lives of the DNA of from 115 to 800 days in the brain of normal adult mice and from 13 to 58 days in heart muscle, smooth muscle, liver, seminal vesicles, mast cells, and interstitial cells in the testis. A survey of the results of various authors is given in Table V and shows reasonable agreement for the slower component of loss. For comparison the biological half-lives calculated from determinations of mitotic

indices are included in Table V. In intestines and other dividing organs the faster component can probably be explained by loss of labelled cells by migration whilst the slow component is likely to be accounted for by labelled cells in the subsidiary tissues. For example, in the small intestine of a mouse killed 1 hour after injection with labelled thymidine, labelled cells are frequently seen in the lamina propria in addition to those in the epithelium of the crypts. At this time most of the activity is in the epithelial cells. Three days after injection, the total content of the epithelial cells is much diminished, indeed more than would be expected from dilution through cell division, whilst the number and grain count of labelled cells in the lamina propria shows little change. The initial rapid fall in activity observed by Garder and Devik (4) in liver, muscle, and kidney is possibly due to a DNA-fraction lost by other workers during preparation for autoradiography or chemical analysis.

These results of investigations in various laboratories show considerable instability of DNA if labelled thymidine is used as a precursor. The autoradiographs always show definite labelling of a small proportion of all nuclei, and therefore widespread chemical turnover which would affect the DNA of all cells can be excluded. Instability of DNA has now been observed in many differentiated organs and it can be considered to be a feature of differentiated cells in higher organisms, as previously suggested by the present author.

In a previous publication (8) cell renewal by a process other than cell division, namely renewal of

TABLE V

Biological Half-Life of DNA Based on Labelled Thymidine in Adult Animals

Reference	*G*	*G*	*D*	*P*	*L*
Method	*loss b*	*inc. b*	*loss b*	*inc. a*	*div*
	days	*days*	*days*	*days*	*days*
Intestine	1.2, 21	0.7	2.8, 19	—	1.3
Liver, normal	14	14	2.8, 25	21	400
Liver, hepat.			480		
Spleen	2.8, 28	3.1	4.2, ?		
Skeletal muscle	17	15	7, 29		∞
Smooth muscle				15	∞
Heart muscle				40	∞
Thymus	1.8, 49	4.2			
Kidney	39	18	4.2, 35		>87
Brain				115	∞
Mast cells				15	
Seminal vesicle				12.5	350
Interstitial cells in testis				43	

G, Gerber *et al.* (5).
D, Garder and Devik (4); Devik and Halvorsen (2).
P, Pelc. (8, 10, and recent results).
L, Leblond and Walker (6), and Pelc and Gahan (10).
loss b, biological half-life was determined by measuring the loss of H^3 by biochemical means.
inc. b, determined by biochemical means and comparison of specific activity shortly after injection in different organs (liver = 14 days).
inc. a, determination by counting the number of labelled cells in autoradiographs.
Some of the data were calculated from published values and can only be taken as approximations. Where two values are given, a biphasic curve indicates initial loss at a faster rate than the subsequent loss at a slower rate.
div, biological half-lives calculated from mitotic rates. >87 denotes that the daily turnover of cells is less than 0.8 per cent (6); ∞ that no divisions were found.

DNA, has been suggested to explain incorporation of DNA precursors not leading to mitosis. The new results presented in this paper add to the evidence supporting this suggestion. Further work will be needed to elucidate the details of this process, such as to determine whether this non-mitotic incorporation of precursors into DNA represents complete re-synthesis of DNA with a temporary increase to 4C values, or breakdown of DNA simultaneous with or preceding synthesis. An interesting possibility might be that the strands of the double helix of DNA separate, one strand being utilised for synthesis of new molecules and the other strand being discarded. In this way a true copy of a 2C amount of DNA could be obtained.

It might be suggested that these findings contradict the current theories of synthesis and biological action of DNA. It seems, however, perfectly possible that parts or all of the DNA of a cell can be renewed by self-reproduction and that DNA still acts as a template. It seems merely necessary to assume that after some time DNA molecules become unusable, similar to the processes generally envisaged for enzyme-molecules.

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