EVIDENCE FOR AN ESSENTIALLY CONSTANT DURATION OF DNA SYNTHESIS IN RENEWING EPITHELIA OF THE ADULT MOUSE

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ABSTRACT

Tritiated thymidine autoradiography has been applied to several renewing epithelial tissues of the adult mouse in order to determine (*a*) the average time required for DNA synthesis; and (*b*) the temporal relationship of the synthesis period to the progenitor cycles of these populations. The average duration of DNA synthesis has been computed from curves describing the rates of appearance and disappearance of labeled metaphase figures in epithelia of colon, ileum, duodenum, esophagus, and oral cavity, in both normal and colchicine-treated animals. In general, application of colchicine does not significantly influence the derived values for DNA synthesis duration. The DNA synthetic time is remarkably similar in the tissues examined, despite wide differences in the times required for completion of the progenitor cycle (and for tissue renewal). Synthesis of DNA in these epithelial cells of the mouse requires approximately 7 hours. Agreement between this value and those derived by other investigators for mammalian cells *in vivo* and *in vitro* indicates that DNA synthetic time may be a temporal constant, of considerable potential utility to studies of cell proliferation. The advantages and shortcomings of this experimental approach to problems of cell population kinetics *in vivo* are discussed.

INTRODUCTION

After cessation of absolute growth in mammals, cells in many tissues and organs maintain their capacity for mitotic division, so providing a continuous and balanced supply of new cells in replacement of those which are presumably no longer functionally competent. Equilibrated cell turnover in the adult is readily demonstrable in epithelial tissues which directly contact the external environment, and for the most part probably reflects local functional demands which the environment places on the cell population; *i.e.*, "wear and tear." Convenient as the "wear and tear" concept is, however, some degree of renewal may be an inherent property of the cell population concerned, more or less independent of environmental demands.

Investigations of the rate at which tissue turnover occurs have been frequent, and have concerned themselves with a wide variety of species and cell types. For the most part, studies undertaken in this vein have involved calculation of the "mitotic index," following administration of the mitotic spindle poison, colchicine. Results of these studies have been reviewed elsewhere (12, 13, 19). They demonstrate that there are wide differences in the rate at which steady-state tissue renewal

occurs, and that within the same type of cell population there are distinctive variations according to species as well.

The recent introduction of tritiated thymidine (hereafter abbreviated H^3-T) for autoradiographic recognition of newly synthesized deoxyribonucleic acid (DNA) has permitted further exploration of the kinetic details of such steady-state cell populations (3, 5, 7, 20, 30, 37, 38). The present study has utilized H^3-T autoradiography in order to define more precisely certain aspects of the steady-state *in vivo*. In particular, attention has been focused on the progenitor cycle and the temporal relationships of DNA synthesis.

Generative or stem cells of an epithelial population are referred to in this paper as *progenitor cells*, as opposed to the bulk of epithelial cells in the population which are differentiated and nonmitotic. The *progenitor cell cycle duration* is, therefore, the average time elapsed between successive divisions of any one of the progenitor cells. By contrast, *tissue turnover time* is defined as the period required for complete replacement of all of the cells in the tissue population, and so is a function of both the progenitor cell cycle duration and the proportion of progenitor cells present in the population.

Representative epithelial populations of the adult mouse were chosen for this study, since they meet the steady-state requirements. The tissues selected were those whose turnover times were known to differ widely. Systematic application of serial sacrifice techniques and autoradiography following administration of H^3-T has permitted: (*a*) determination of the DNA "*synthetic index*," *i.e.*, the relative proportion of progenitor cells which have utilized H^3-T at an instant in time; (*b*) derivation of the average time required for cellular synthesis of DNA; and (*c*) calculation of the progenitor cell cycle duration.

Our results reveal the existence of a basic similarity among the various epithelial populations in regard to the duration of the DNA synthetic phase.

MATERIALS AND METHODS

Two experimental procedures were undertaken, one involving combined administration of H^3-T and colchicine, the other, administration of H^3-T only. Subsequent analysis of the resulting data revealed, however, that differences introduced by colchicine treatment were of no appreciable consequence in most instances.

Experiment 1. Colchicine and Tritiated Thymidine

Eighteen 60 day old Swiss albino mice, averaging 29 gm in body weight, were utilized. Of these, eleven were males, and seven were females in the 11th day of gestation. All animals were sacrificed by exsanguination under ether at the same hour (2:30 p.m.) to minimize the effects of diurnal variation on cell proliferation rate. Four hours before sacrifice each animal received a single intraperitoneal injection of colchicine (Eli Lilly and Company, Indianapolis, 10 mg per cent solution in normal saline) in a dose of 1 mg/kg body weight. At intervals ranging from 45 minutes to 30 hours prior to sacrifice, each of the males was injected intraperitoneally with 10 μc H^3-T (Schwarz BioResearch, Inc., Orangeburg, New York, specific activity 0.89 c/mM) in 0.2 cc distilled water. The females also received 10 μc H^3-T in the same manner, but at intervals ranging from $1\frac{1}{2}$ to 18 hours prior to sacrifice.

Experiment 2. Tritiated Thymidine

Eighteen male Swiss albino mice, 70 days of age and averaging 30 gm body weight, were utilized in this experiment. At 2:00 p.m., each animal received a single subcutaneous injection of 10 μc H^3-T (specific activity 0.36 c/mM) in 0.2 normal saline. The animals were sacrificed in pairs, by a blow on the head, at nine intervals ranging from $\frac{1}{4}$ hour to 14 hours following injection.

Subsequent procedures were similar in both experiments. Representative portions of epithelium from buccal mucosa, tongue, esophagus, duodenum, lower ileum, and descending colon were immediately fixed in Hollande's modification of Bouin's fluid, or in Carnoy's fluid. After fixation they were embedded in paraffin and were sectioned at 6 microns. Alternate sections were mounted on slides and autoradiographed according to the dipping method described by Messier and Leblond (24). Preparations were stained either by the Feulgen technique prior to autoradiography, or by Harris' hematoxylin after exposure and development. Eastman Kodak NTB-2 bulk emulsion was utilized for autoradiography, with exposure times ranging from 10 days to 3 weeks at 4°C.

The autoradiograms were examined under oil immersion at 1,125 diameters in order to determine: (*a*) the percentage of metaphase figures which were labeled at the various times of sacrifice; and (*b*) the percentage of germinal or progenitor cells which were labeled in each type of epithelium at an early interval after H^3-T administration. A total of 100 metaphase figures were counted in each tissue to establish the first point, while 1,000 labeled and unlabeled cells were counted in each tissue to estab-

lish the second. Our analyses were restricted to those portions of the epithelial tissues wherein mitoses were known to occur. The cells in these regions were considered to constitute the progenitor population. These consisted of: the cells of the basal layer of the oral, lingual, or esophageal epithelium; the cells lining the intermediate two-thirds of the sides of the crypts of Lieberkuhn in the lower ileum and duodenum; and, in the descending colon, the cells lining the basal portions of the crypts.

RESULTS

For convenience, the *progenitor cycle* has previously been divided into four phases (14) as illustrated in Fig. 1. These are: M, the mitotic phase; G_1, the initial portion of interphase during which no synthesis of DNA occurs; S, the period of DNA synthesis; and G_2, the post-DNA synthetic, terminal portion of interphase. If equilibrium is to be maintained in a renewing cell population, on the average, each mitotic division of a progenitor cell is followed by the return of one daughter cell to the progenitor cell pool, while the other leaves the pool and differentiates to replace a cell which has been lost (Fig. 1).

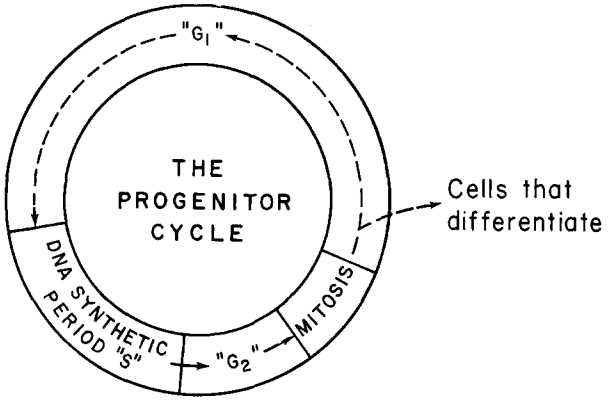

FIGURE 1
Diagrammatic representation of the progenitor cycle of a renewing cell population. The diverging arrow indicates that some of the cells produced do not return to the cycle (see text).

It is now generally accepted that the incorporation of H^3-T into cells is strictly indicative of DNA synthesis (9, 31), and it is usually assumed that the labeled cells are metabolically and environmentally homologous to their unlabeled fellows (20). It is also generally agreed that H^3-T is available in the circulation only for a short period of time after its administration and that there is no endogenous pool of thymidine normally present in the body (22, 29, 30, 32). This being the case, one may safely assume that those cells which are labeled at any time after injection of H^3-T must be those which were in the S phase of the progenitor cycle at the time of injection, or be the progeny of such cells.

Kinetics of Labeled Cells in Metaphase

Tissues normally possessing a slow turnover time demonstrate a corresponding paucity of mitotic figures at any instant. Since metaphase figures were necessary to the analysis at hand, one series of experimental animals received colchicine to arrest and accumulate dividing cells in the metaphase stage. In the other experimental group, however, no colchicine was used, but sufficient numbers of autoradiograms were prepared and examined to permit valid analysis of the metaphase labeling.

Curves describing the rate of accumulation of labeled metaphase figures were prepared, typical examples of which are illustrated in Fig. 2. Here, the percentage of labeled metaphase figures in the epithelia of duodenum and esophagus is plotted as a function of the time between H^3-T administration and sacrifice. Two curves are shown in each of these graphs, representing respectively, the figures obtained following combined administration of colchicine and H^3-T (solid line), and those obtained after administration of H^3-T alone (broken line). Examination of the curves reveals that the progress of labeled cells into mitosis occurred quite rapidly in both epithelia. However, depending upon whether colchicine was employed and upon the epithelial population concerned, the rate at which labeled metaphase figures accumulated varied.

In the duodenum, labeled metaphase figures could be detected as early as ¾ hour after H^3-T

administration alone, or at 1 hour with prior colchicine treatment. In the non-colchicine group, however, virtually the entire metaphase population became labeled within 2 hours of H^3-T administration, while following colchicine nearly 5 hours elapsed before 100 per cent of the metaphase figures was labeled. In the esophagus, this general pattern was repeated, although its timing was

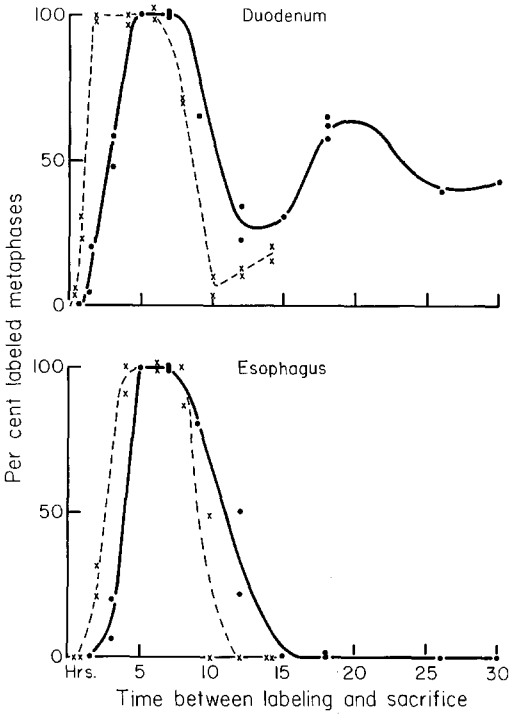

FIGURE 2

Curves demonstrating the percentage of labeled metaphase figures in the epithelia of duodenum (above) and esophagus (below) plotted as a function of the time between H^3-T administration and sacrifice. Each symbol represents a single animal. Solid line curves and dashed curves represent, respectively, results obtained in epithelium with and without prior colchicine treatment.

different. Thus, nearly 2 hours elapsed before labeled metaphase figures could be detected in the non-colchicine group, and only at 3 hours after H^3-T administration did they appear in the colchicine-treated group. Similarly, a period of between 3 and 4 hours was required before the 100 per cent level of metaphase labeling was achieved in the non-colchicine group, as opposed to nearly 5 hours in animals treated with colchicine. A comparable pattern of metaphase labeling and colchicine influence thereon was observed in all of the epithelial populations examined in this study.

The 100 per cent level of metaphase labeling was maintained in all the epithelia for a variable period, again apparently depending on the tissue in question and on prior colchicine treatment. Following this plateau phase, the percentage of labeled metaphases declined with time, more rapidly in the non-colchicine groups than in tissues affected by colchicine (Fig. 2).

The differences between the colchicine and non-colchicine curves may be accounted for as follows. In the colchicine-treated group, the drug was administered 4 hours prior to sacrifice in all cases. In those animals sacrificed soon after injection of H^3-T, therefore, colchicine had already caused unlabeled metaphase figures to accumulate in numbers out of proportion to the normal, and thus diluted the incoming population of labeled metaphases. Hence, the slope of the ascending portion of a colchicine curve was not so steep as that of the non-colchicine curve. On the other hand, in animals sacrificed at later intervals after H^3-T administration, the colchicine effect was directed at a population of already labeled cells, and caused the accumulation of considerably more labeled than unlabeled figures. Therefore, the slope of the descending limb of a colchicine curve was also less marked than that of the corresponding non-colchicine curve. Comparison of colchicine and non-colchicine curves demonstrates that the action of the drug was to impede, more or less equally, the rates of both appearance and disappearance of labeled metaphase figures. However, the shapes of the two curves were sufficiently alike to serve for the calculations which follow.

Determination of Mean DNA Synthetic Time

The duration of the *S* phase of the progenitor cycle can be calculated by quantitative assessment of the time course of appearance and disappearance of labeled cells in any particular stage of *M*, the most readily recognizable of which is metaphase. In this study, therefore, the DNA synthetic time has been calculated from curves of the type illustrated in Fig. 2, derived for each of the epithelial populations examined.

Biological variation among individual cells within the population under scrutiny, combined with the difficulty of detecting weakly labeled

metaphase figures by autoradiography, greatly reduces the precision and significance of integrative measurements of entire curves such as those illustrated in Fig. 2. It has been suggested, however, that the mean value of DNA synthesis duration in a given progenitor population is accurately represented by the interval between the time that a 50 per cent level of metaphase labeling has been attained soon after administration of H^3-T, and the time that the 50 per cent level is again reached at a later interval. From the practical point of view, moreover, this method of estimation tends to minimize errors which may have been introduced by biological variation among individual cells or by deficiencies in autoradiographic sensitivity. The method was introduced by Quastler and Sherman (30) and has been used subsequently by many others (3, 5, 15, 23, 25, 27, 37, 38). Table I presents the results of our estimates of the mean value of *S*, obtained in this manner, for each of the epithelial populations examined in this study.

TABLE I

Mean Duration of DNA Synthesis

Type of epithelium	Colchicine-treated animals	Normal animals
	hrs.	*hrs.*
Buccal mucosa	7.2	—
Esophagus	7.0	6.9
Tongue	—	7.0
Duodenum	7.4	7.4
Lower ileum	7.4	7.1
Colon	8.2	7.5

The mean duration of *S* in the normal animal approximates 7 hours for all of the epithelial populations studied. This result is in strikingly good agreement with estimates made by others, both *in vivo* and *in vitro*, for a variety of cell populations (Table III). Prior administration of colchicine did not materially alter this estimate of *S* duration, except in the case of the colonic epithelium. This discrepancy is discussed below.

Determination of Mean Progenitor Cycle Duration

The percentage of interphase cells labeled at ¾ hour after H^3-T injection is a reliable indicator of the relative size of the progenitor population in the *S* phase, *i.e.* the *synthetic index*, assuming as before that H^3-T is available in the circulation for only a short time. Within a 45 minute period, it is most unlikely that a cell could have become labeled and also have completed mitosis. At a later interval the apparent size of the DNA-synthesizing progenitor population would be artificially increased by the inclusion of labeled daughter cells. Counts of labeled and unlabeled interphase progenitor cells were made for the various epithelial populations, and the synthetic index for each at 45 minutes after H^3-T injection is listed in Table II.

Calculation of mean progenitor cycle time was made according to the method suggested by Quastler and Sherman (30), and is generally analogous to the technique for determining tissue renewal time from the mitotic index. Thus, if *n* is the number of cells in the DNA synthetic phase (*S*) at an early interval after H^3-T administration, *N* is the total number of progenitor cells, *t* is the mean duration of the *S* phase, and *T* is the mean duration of the entire progenitor cycle, then:

$$\frac{n}{N} = \frac{t}{T}$$

By application of this relationship, the mean progenitor cycle durations of the various epithelial populations were calculated and are also listed in Table II. These data provide convincing evidence that the duration of DNA synthesis is quite independent of the duration of the progenitor cycle of the particular population involved.

TABLE II

Mean Progenitor Cycle Duration

Type of epithelium	Cells in DNA synthesis at ¾ hr.	Calculated progenitor cycle time	
		Colchicine-treated	Normal
	per cent	*hrs.*	*hrs.*
Buccal mucosa	8.5	85.0	—
Esophagus	3.8	184.0	181.0
Tongue	18.0	—	40.0
Duodenum	40.0	18.5	18.5
Lower ileum	42.0	17.5	16.7
Colon	23.0	35.6	32.6

TABLE III

Duration of DNA Synthesis Phase (from Literature) and Calculated Cell Generation Time in Mammalian Cells*

Cell system	Reference	Mean DNA synthetic time	Calculated cell generation time‡
		hrs.	*hrs.*
In vitro:			
L-strain cells	(35)	6–7	20
HeLa S3 cells	(26)	6	25
Hamster cells	(36)	6	14
Ascites tumor cells	(8)	7.5	18
Liver cells	(18)	8	31
L-5178Y ascites cells	(6)	6.9–7.4	—
HeLa cells	(26)	8.5	28
In vivo:			
Human bone marrow	(5)	6.5	—
Canine myelocyte series	(27)	*ca.* 5	16–18
Canine myeloblasts	(27)	*ca.* 5	9
Mouse ileum	(30)	7.5	18–19
Mouse hair follicle	(3)	7.5	12
Mouse breast cancer	(23)	10	24–72
Mouse tongue	(37)	8.5	96
Rat hepatocytes	(22)	8	—
Rat chondrocytes	(16)	8.5	—
Rat bone cells:	(38)		
Metaphysis		8	36
Endosteum		8	57
Periosteum		8	114
BNL tumor	(15)	8	16

* This listing should be considered representative rather than comprehensive. Recently, the frequency of reports regarding DNA synthesis time has greatly increased. For the most part, however, there is agreement that the duration of DNA synthesis is from 6 to 8 hours.

‡ Based on estimate of "synthetic index" provided by the reference, where given.

DISCUSSION

The introduction of tritiated thymidine autoradiography has served to facilitate examination of renewing cell populations, and at the same time has tended to focus attention on that aspect of tissue turnover which is probably of the greatest significance in terms of renewal regulation, namely the progenitor cell cycle. While tissue turnover time in a particular system is a reflection of both the progenitor cell cycle duration and the size of the progenitor pool, it is probable that the former serves as the primary target for the various stimuli which normally regulate cell proliferation and tissue turnover.

By tracing the serial progression of labeled progenitor cells from the DNA synthetic phase (*S*) through mitosis, it has been possible to measure both the duration of the *S* phase and the duration of the progenitor cycle in each of various adult epithelial populations. The most striking finding in the present study is the apparent constancy of the duration of *S*, irrespective of the time required to complete the progenitor cycle. The agreement between the values of *S* reported here and their similarity to estimates made by other investigators for mammalian cell types both *in vivo* and *in vitro* (Table III) suggests that the duration of the DNA synthetic phase may be identical in all epithelial cell populations of the adult mammal. Intimations to this effect have already appeared in the literature (6, 8, 33); but it must be admitted that full confirmation of the ubiquity of a constant duration of *S* requires further investigation, since there are also reports to the contrary in connection with studies of the testis (4, 11, 25) and ear epidermis (34).

Validity of the Measurement of S

While it is not necessarily pertinent to the interpretation of the results reported here, it seems appropriate to emphasize that the difficulty of delimiting a progenitor population *in vivo* is potentially the most important methodological shortcoming in this experimental approach to the study of progenitor populations. The data from the analysis of the colonic epithelium, for example, reveal a discrepancy between the calculated values of *S* in normal and colchicine-treated groups. It does not appear that this difference has stemmed from an error in experimental observation and, indeed, it may reflect the normal variation possible in synthesis time. On theoretical grounds, however, it also seems possible that this error was introduced by improper selection of a colonic progenitor population.

It has been noted previously that our definition of the morphological distribution of a cohort of

progenitor cells was based entirely on histological distribution of mitotic activity. It was assumed that all dividing cells belonged to the same progenitor population. Such an assumption is more readily defensible in the case of stratified epithelia than it is in the case of intestine. In the latter, several distinct types of differentiated cells are found, which may or may not necessarily arise from the same progenitor population. In the duodenum, for example, fully differentiated cells of Paneth at the bases of the crypts exhibit mitotic activity which is apparently independent of that observed in relatively undifferentiated cells lining the sides of the crypts. Consequently, our observations of duodenum did not include the bases of the crypts but were restricted to cells lining the sides of the crypts. There is no comparable basis for selecting a colonic progenitor population. If, however, the colonic epithelium possessed two progenitor populations of dissimilar cycle time, as a recent report has suggested (21), the curve describing the rate of appearance and disappearance of labeled metaphase cells would include both.

It has already been noted that the lag time between administration of H^3-T and the first appearance of labeled metaphase figures may vary considerably between different progenitor populations (in Fig. 2 compare broken-line graphs of normal duodenum and esophagus). This lag phase, which represents the sum of the durations of G_2 and prophase, tends to relate more or less directly to the duration of the progenitor cycle (2). Calculations based on a model of such a bipartite progenitor population indicate that differences in the relative lengths of the $G_2 + P$ periods of the two populations will not materially alter the shape of the composite curve demonstrating the rate of accumulation of labeled metaphases in *normal* animals. However, similar calculations which assume *colchicine* treatment 4 hours prior to sacrifice (as in the present study) suggest that differences in the $G_2 + P$ interval may be reflected in the shape of the resulting composite curve, so as to increase or decrease the apparent duration of S measured in the manner outlined above. While further study is required as to possible influences of this type on our data from the colon, the foregoing comments emphasize the necessity for careful definition of a progenitor population prior to its investigation by this technique.

Application of the Value of S to Renewing Systems

The relationship between tissue turnover time and the normal or experimental factors which may operate in regulating it has been assayed in the past by means of the mitotic index. This figure is derived from the relative number of mitoses identified in a statistically large sample of cells, with or without prior colchicine treatment. The technique is somewhat tedious, and, more importantly, the introduction of colchicine may bias the ultimate calculation of turnover time values (compare values in Table I, reference 19). Finally, as sometimes applied, the method depends on an assumed value for the duration of mitosis, which seldom lends itself to precise definition and is known to vary from tissue to tissue (2, 13, 19).

By contrast, the apparent invariability of DNA synthesis duration could provide a valuable "unit constant" for analytical treatment of renewing populations. A constant value of S, coupled with determination of the synthetic index, would permit a straightforward technique for determining progenitor cycle durations and, indeed, tissue turnover times. If the constant duration of S were assumed, it would be possible to calculate progenitor cycle times in a variety of renewing populations by determining their synthetic indices at a single, early time interval after H^3-T administration. Tissue turnover time could also be computed by relating the synthetic index to the total cell population rather than to the progenitor population.

Lastly, it should be noted that at early intervals after H^3-T administration labeled cells are in the interphase stage of the progenitor cycle. Appropriate autoradiographic technique will consequently allow a more precise morphological classification of cell types in the progenitor pool than would be the case if only mitotic figures could be utilized. Moreover, since the duration of the S period is some 7 hours while that of mitosis is usually presumed to be about 1 hour, the "synthetic index" is a sevenfold more sensitive assay for proliferative activity.

Regulation of DNA Synthesis and the Progenitor Cycle Duration

Despite wide variations in progenitor cycle time and tissue turnover time, the duration of S

derived in this and many other studies appears to be constant. Definition of the time required for DNA synthesis *in vivo*, as presented here, introduces the possibility of investigating the nature of those biochemical and other factors which seem to regulate it so precisely. Perhaps the most obvious speculation which could be made in this connection is that the time required for DNA synthesis is a direct function of the amount of DNA which must be replicated. Since the duration of S of 7 hours has been calculated for diploid somatic cells, each of which contains the same amount of DNA, it would be of interest to know what result would be obtained under polyploid conditions, or in cell systems which contain less than the mammalian complement of DNA. Work along these lines has been undertaken and will be reported subsequently. Our preliminary data, however, suggest that the quantity of DNA per cell does not relate directly to synthesis time.

The ability to determine progenitor cycle duration according to the technique outlined above also offers prospects for elucidation of many aspects of cell population kinetics which heretofore have not lent themselves to convenient analysis. As an example, it should be quite feasible to study the effects of various environmental or exogenous stimuli on the progenitor cycle duration. With respect to environmental influences, it has been suggested that they may alter the duration of the cycle through changes in the duration of the post-DNA synthetic (G_2) phase (10). However, undoubtedly the greatest flexibility in the cycle lies in the G_1 portion of interphase, as others have indicated (23, 38). Some of our current studies have been directed at determining progenitor cycle durations in embryonic and perinatal cell populations. It has been possible to demonstrate that the duration of the progenitor cycle in such populations is markedly less than it is in the adult. In some embryonic epithelial populations, in fact, preliminary investigation indicates that the progenitor cycle duration is only slightly longer than the time required for the combined processes of DNA synthesis and mitosis. In these systems, therefore, the G_1 phase of the progenitor cycle is virtually non-existent.

Addendum

Just as this manuscript was completed, the report of Koburg and Maurer (17) appeared in the literature. Using autoradiographic grain counts, these authors also concluded that the duration of DNA synthesis in diploid mammalian cells lay in the range of 7 to 8 hours. Very recently, also, this group of investigators has introduced a novel technique for determining DNA synthesis involving two spaced injections of H^3-T or of H^3-T and C^{14}-T. By means of this technique they have confirmed the extended DNA synthesis duration exhibited by rat spermatogonia (11) already noted by others (4, 25). However, they have also shown that DNA synthesis time in all other cell populations of rat and mouse which were examined possesses a mean value of 7.5 hours (28).

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