Cell proliferation and differentiation in the human epidermis have been studied by KAKU, IGARASHI and FUJITA (1964) using the method of “single pulse-migration chase” (FUJITA and MIYAKE 1964, FUJITA 1965). Labeled cells first appeared in the deeper layers of the epidermis and, as time elapsed, migrated upward until they reached the surface of the Malpighian layer 21 days after the pulse labeling. A generation time of 4.2 days was estimated for the generative cell and a life span of 21 days for the differentiated cell in the spinous and granular layers. Thus continuous cell turnover was confirmed in the human epidermis. New cells are produced in the deeper layers of the epidermis and some of them migrate upward and are sloughed off. The number of cells produced in the deeper generative zone and of those falling off from the surface are precisely balanced so that a steady state is maintained.

The commonly accepted explanation for the mechanism of steady state turnover is to assume the presence of unequal divisions or (G, M) divisions in the generative cell (COWDRY 1950, OSGOOD 1959, OEHLERT, COTE and BÜCHNER 1961). According to this theory, one daughter cell is decided to differentiate at the time of mitosis and another to remain as the generative cell. The theory has been universally applied to explain the mechanism of constantly renewing cell systems and regarded as one of the most basic principles of biology. However, FUJITA and MIYAKE (1964), FUJITA (1965) investigated various systems of cell differentiation, i.e. the steady state of intestinal epithelium, the healing process of the lens endothelium and the development of neuroblasts in the neural tube (FUJITA 1963), and concluded that types of mitosis can not determine cell differentiation but that two daughter cells are equivalent at least shortly after division. Cell differentiation should take place during t1 or G period of the newly formed daughter cell. Independently MARQUES-PEREIRA and LEBLOND (1965) studied cell differentiation in the squamous epithelium of the rat esophagus and came to a similar conclusion.

The aim of the present work is to complete our previous work on the human epidermis (KAKU, IGARASHI and FUJITA 1964) and to analyse the mechanism of cell differentiation in this system by means of various techniques of ³H-thymidine auto-
radiography. In the present paper, special emphasis is laid on the elucidation of the correlation between the proliferation and the differentiation of the epidermal cells, since the two phenomena are intimately coupled in the actual system, and forced separation would result in one-sided view of the dynamic mechanism of the organism.

**Materials and Methods**

The skins used in this study were those covering mammary glands which were decided to be removed by a radical operation of carcinoma. A small amount (5 μC) of ³H-thymidine with specific activity of 5.0 C/mM [thymidine (methyl-T), the Radiochemical Center] was dissolved in 0.1 ml of 5% glucose solution and injected intradermally to produce a small papule. The elevation disappeared within 30 minutes but ³H-thymidine seemed not to diffuse out beyond the area originally occupied by the papule; the label was incorporated in the epidermal cells that are located within the limit of the original papule. This fact assured, on the one hand, the safe application of this local labeling method to the human tissue *in vivo* since the radioactive substance was removed almost completely from the human body by a subsequent surgical procedure. But the limited diffusion of the isotope, on the other hand, produced a technical difficulty since the paraffin section used for the autoradiograph should include this small portion. The difficulty multiplied when repeated injections were required for carrying out cumulative labeling; all the injections should hit the same target. Special attention was thus necessary to obtain the effect of the continuous labeling.

Two patients with breast cancer were used for this experiment, case 1 being a 42 year old woman and case 2, a 43 year old one. On the skin of the mammary gland of the case 1 patient, nine points were marked with magic ink and of the case 2, eight points. These points were separated from each other by at least 2 cm. Eight points of the case 1 were selected to carry out flash and single pulse labeling. Each point was injected with ³H-thymidine 192.5, 168, 116.5, 89, 67, 44, 19 and 3 hours before the operation. One point received one injection only and no multiple injection was made. This group of 8 points served as flash and single pulse-migration chase. The remaining one point was injected with the label repeatedly at intervals of from 4 to 11 hours, the total duration of the cumulative labeling being 3.7 days or 89 hours. Originally the interval of 8 hours was intended but the schedule was forced to change by the patient's waking-sleeping cycle. This point served as cumulative labeling. In case 2, all the eight points were used to carry out flash and single pulse-migration chase experiments. Each point was injected respectively with ³H-thymidine 112, 88, 65, 43, 23, 4.5, 3.5 and 2.5 hours before the operation. In both cases, the skin including all the labeled points was removed at once at the time of the operation and the stripped skin was immediately fixed in Carnoy's fluid. After cutting into separate blocks, each containing one point of labeling, the tissue was embedded in paraffin, cut at 5 μ and autoradiographed by dipping into Kodak NTB 3 or Sakura NR-M2 emulsion or by the stripping (Fuji ET-2E stripping film) method.
Results and Interpretation

Flash label autoradiography

Flash autoradiographs were obtained 3 and 2.5 hours after the local labeling, in case 1 and 2, respectively (Fig. 1). In both cases the epidermis was composed of 3—5 layers of Malpighian cells. The deepest layer contained many pigmented cells. In the deeper two layers, cells were packed rather densely but the more superficial 2—3 layers were less crowded. The deepest layer, adjacent to the dermis is the basal layer and the layer directly above the basal layer is called the epibasal layer. Contrary to our expectation, the flash label autoradiographs showed rare labeled cells among pigmented cell population and labeled cells were more frequent in the epibasal layer than in the deepest basal layer. This tendency was more conspicuous in case 1 in which melanin containing cells were more abundant than in case 2. This finding reveals considerable difference in the structure of the human epidermis from the squamous epithelium of the mouse and rat; SHERMAN, QUASTLER and WIMBER (1961), OEHLERT, COTE and BüCHNER (1961), WOLFSBERG (1964), MARQUES-PEREIRA and LEBLOND (1965), and FRANKFURT (1967) found flash label cells only in the basal layer in the squamous epithelium of mouse and rat. Our finding in the human epidermis indicates, (1) that the melanin containing cells constitute a population kinetically different from other generative cells in the basal layer, their turnover being much slower, and (2) that the stratum germinativum or G-zone of the human epidermis does not correspond to the basal layer alone but to the epibasal and the basal layers with an emphasis on the former. This double-layered generative system in the human epidermis complicates the analysis of cell proliferation and differentiation in this structure.

Fig. 1. Flash label cells in the human epidermis, of a 42 year old woman. Autoradiograph taken 3 hours after an injection of 3H-thymidine. The labeled cells are located in the basal (left) and in the epibasal (right) layer. No labeled cells are found in the superficial layers. × 800
The percentage of labeled cells or labeling index was measured by counting the number of labeled cells in the epidermis and dividing the number by the total number of cells in the epibasal layer. Generative cells in the basal layer were not included in the count. The labeling index counted in this way was 5.2% and 5.0% respectively in case 1 and case 2.

Cumulative label autoradiography

In case 1, repeated injections of \(^{3}\)H-thymidine up to 11 times have been made in the same region. The total duration of the continuous labeling was 3.7 days or 89 hours. The interval of 8 hours was aimed at first since we know that DNA synthesis time or \(t_c\) in the human epidermis is approximately 7 hours (KAKU, IGARASHI and FUJITA 1964). To obtain an effect of continuous labeling, \(^{3}\)H-thymidine should be given every 7 hours or at shorter intervals. But, since injected thymidine remains in the tissue for 1 hour or longer, we estimated the needed intervals of injection to be at a minimum of 8 hours. Unfortunately, we could not observe the 8 hours’ intervals, since the patient sometimes slept early and woke late. Consequently the intervals ranged from 6 to 11 hours. The delay of some of the injections seemed to be reflected in the result by the appearance of some unlabeled cells in the epibasal layer (Fig. 2).

Autoradiographs of the cumulatively labeled epidermis visualized the presence of many proliferating cells in the epibasal layer and relative paucity in the basal layer.

Fig. 2. Cumulative label autoradiograph of the human epidermis of a 42 year old woman. \(^{3}\)H-thymidine was repeatedly injected for 3.7 days. Most cells in the epibasal layer have incorporated heavy label. Several cells in the basal layer are also labeled. Exceptionally a few unlabeled cells (indicated by an arrow) are found in the epibasal layer. Superficial cells are not labeled. \(\times 400\)
(Fig. 2, 3). Most melanin containing cells showed no label as seen in Figure 3. In contrast, most cells in the epibasal layer incorporated heavy label. Most cells in the population in the epibasal layer had passed through DNA synthetic phase during the cumulative labeling. Close examination of the autoradiograph, however, revealed a few unlabeled cells in the epibasal layer (arrow in Fig. 2). The authors regard these cold cells as cells that happened to synthesize DNA when the injection of $^3$H-thymidine was delayed for several hours. However, an alternative explanation is possible. It has been shown, (FUJITA 1962) that, to accomplish 100% labeling of a population, it is necessary to keep the label available as long as $t_G + t_M + t_i = t_G + t_S$. Therefore, one may assume that the presence of the unlabeled cells is the result of shorter duration of the cumulative labeling which could not cover the sum of $t_G$, $t_M$ and $t_i$ durations of the generative cell. However, unlabeled cells in the basal layer are not so many and it is possible to consider that all the cells in the G-zone i.e. cells in the epibasal layer and non-pigmented basal cells have been labeled during the 87 hours of continuous labeling. We regard these labeled cells as generative cells of the human epidermis and those unlabeled ones as differentiated cells. If we assume that the generative cells are homogeneous and asynchronous population, the time of 100% labeling can be regarded as representing $(t_G - t_S)$ of the generative cell (FUJITA 1962). Therefore, adding 7 hours of DNA synthetic period of the human epidermis (KAKU, IGARASHI and FUJITA 1964) to the time of 100% labeling, i.e. to the 87 hours, we obtain a generation time of 96 hours i.e. 4 days for the generative cells of the epidermis. This estimate is approximately equal to the 4.2 days that were obtained as a generation time for the human epidermis by KAKU, IGARASHI and FUJITA (1964) by the method of single pulse-migration chase. Interestingly the cumulative label autoradiography revealed that the pigmented cells are a kind of differentiated cells though they are in the basal layer, and that proliferating cells predominate in the epibasal layer rather than in the basal layer (Fig. 2, 3). In our unpublished work, we observed that the pigmented cells in the basal layer are able to return to the proliferative state when they are stimulated. In activated forms, they do not contain melanin and their nuclei are swollen. Therefore, the pigmented cells in the basal layer are minor-differentiated cells (FUJITA 1965).
As described above, the epidermal cells proliferate in the epibasal and basal layers and most differentiated cells ascend toward the surface and a few accumulate in the basal layer as pigmented cells. During the 4 days of the cumulative labeling (Fig. 2), several labeled cells have moved out from the G-zone to the upper layer. Therefore, disregarding differentiation into the pigmented cells which are less numerous, we can regard the upward movement of a labeled nucleus from the G-zone as a phenomenon closely related to cell differentiation. From analogy to cell differentiation in the intestinal epithelium, we infer that cells migrated out from the generative layers are either differentiated or destined to differentiate. To find out the rate of production of differentiating cells in the course of time, we counted pairs of

**Fig. 4, 5, 6.** Autoradiograph taken 8 days after a single injection of $^3$H-thymidine, 42 year old woman.

**Fig. 5.** $gg$ pair of labeled cells. $\times 1000$

**Fig. 6.** $gm$ pair of labeled cells. $\times 800$

**Fig. 7.** $mm$ pair of labeled cells. $\times 800$

**Single pulse-migration chase experiments**

As described above, the epidermal cells proliferate in the epibasal and basal layers and most differentiated cells ascend toward the surface and a few accumulate in the basal layer as pigmented cells. During the 4 days of the cumulative labeling (Fig. 2), several labeled cells have moved out from the G-zone to the upper layer. Therefore, disregarding differentiation into the pigmented cells which are less numerous, we can regard the upward movement of a labeled nucleus from the G-zone as a phenomenon closely related to cell differentiation. From analogy to cell differentiation in the intestinal epithelium, we infer that cells migrated out from the generative layers are either differentiated or destined to differentiate. To find out the rate of production of differentiating cells in the course of time, we counted pairs of
labeled cells of which one or two members migrated upward out from the generative zone at various time intervals after the pulse labeling with $^3$H-thymidine, as Marques-Pereira and Lefblond (1965) carried out. Pairs of the labeled cells can be classified into 4 types (Fig. 4, 5, 6). Suppose $g$ and $m$ represent undifferentiated and differentiated daughter cell respectively, the 4 types of pairs can be described as $gg$ or $g^2$, $mg$, $gm$, and $mm$ or $m^2$. Since we can not distinguish $mg$ and $gm$, they may be practically regarded as three types as $g^2$, $2mg$ and $m^2$. By $g^2$-state, all the pairs whose both members stay in the epibasal and basal layers are represented, since proliferative cells or labeled cells in these two layers should be regarded as generative cells. Examples of the three types of labeled pairs are illustrated in Figures 4, 5 and 6. Actual counting of these pairs was carried out at various intervals after the pulse labeling and the results are summarized in Figure 7.

### Observed

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<td>2</td>
<td>10</td>
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<tr>
<td>3 days</td>
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### Expected

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**Fig. 7.** Relative frequency of $gg$, $2gm$ and $mm$ pairs. Actual observation and theoretical expectation. Left: Ratios actually observed in the epidermis of a 42 year old woman at 1, 3, 4, and 8 days after a single injection of $^3$H-thymidine. Right: Theoretical ratios derived from Model I, II and III. For further explanation, see the text.

**Discussion**

**Mechanism of cell differentiation in the human epidermis**

To explain the steady state of cell renewal in the human epidermis in which
production of differentiated cells and regeneration of undifferentiated cells are well balanced, we may construct three different types of models.

**Model I.** In this model it is assumed that the cell differentiation in the steady state is realized by unequal mitoses or \((G, M)\) divisions of the generative cell. When a cell divides, one daughter cell is differentiated and another remains in the same state as the mother cell was. This type of model has been postulated for explaining the steady state equilibrium in the intestinal epithelium (Blues 1956), in the hematopoietic system (Weicker 1954, 1956), in the mouse skin (Oehlerlert, Cote and Büchner 1961), and so on. Cowdry (1950) and Osgood (1959) developed extensive theories to explain cell differentiation in the steady state, applying this type of model. If this is the case in the human epidermis, all the pairs of labeled cells should be \(mg\) or \(gm\), and no \(g^2\) or \(m^2\) would occur. Thus the ratio of \(g^2 : 2gm : m^2\) is expected to be 0 : 1 : 0 (see Fig. 7).

**Model II.** In the second model, the steady state of the cell renewal is maintained by equal numbers of \((G, G)\) and \((M, M)\) divisions of the generative cell. One mitosis yields two generative cells and another, two differentiated cells. This type of model is based on the assumption that all the mitoses are made up of equal divisions yielding two equivalent daughter cells. In this model, unequal division does not occur and the ratio of \(g^2 : 2gm : m^2\) is expected to be 1/2 : 0 : 1/2. Both models I and II share one assumption in common, i.e. they implicate that cell differentiation is determined by types of mitosis. To understand the mechanism of cell differentiation this implication is of great importance. If this is true, the determinant of cell differentiation should be sought in the mitosis, although knowledge of modern molecular biology appears to exclude any differential nature of the mitosis itself.

**Model III.** In contrast to the preceding two models, this model does not implicate relationship between mitosis and cell differentiation but assumes that the cell differentiation is determined individually in a daughter cell by chance. If the cell differentiation is a random process subjected only to the rule of probability, the relative frequency of three types of divisions should be given by \(g^2 : 2gm : m^2\), being \(g = 1/2\), and \(m = 1/2\) in the steady state. Here, \(g\) and \(m\) are regarded as probabilities for a daughter cell to be induced into the generative cell or into the differentiated cell, respectively. Therefore, based on this model, the ratio of \(g^2 : 2gm : m^2\) is expected to be 1/4 : 1/2 : 1/4. Theoretically expected ratios are shown in the right column of Figure 7.

If one compares observed ratios in the left column of Figure 7 with the expected ratios in the right column, one may notice that no observed data completely agrees with any expected ones. However, it is clear that the third model gives the theoretical ratio that fits satisfactorily in to the observed data. The expected ratios derived from the models I and II do not resemble to the observed at any time intervals. It is, therefore, concluded that determination of the cell differentiation is independent of types of cell divisions, but that determination of differentiation in the daughter cell is individually subjected to probability, in the steady state \(g\) and \(m\) being equally 1/2.

Similar observation that the cell differentiation takes place by chance during \(t_1\) or \(G_1\) period of newly produced daughter cell, have been made in the steady state
of cell renewal in the intestinal epithelium (Quastler and Sherman 1959, Kaku, Kojima, Hayashi, Horii, Nakamura and Fujita 1962, Fujita 1965, Cairnie, Lamerton and Steel 1965), in the squamous epithelium of the rat esophagus (Marques-Pereira and Leblond 1965), in the regeneration of the lens endothelium of the rabbit (Fujita and Miyake 1964, Fujita 1965) and in the development of neuroblasts in the chick neural tube (Fujita 1963, 1965). The authors have searched for evidence that the differential division might be the cause of cell differentiation but no convincing evidence to support this idea has been found.

Differentiating cells in the epibasal layer

In the present experiments, however, the observed ratio of \( g^2 : 2gm : m^2 \) only approaches to but does not reach the expected values even 4 or 8 days after the pulse labeling. If the epibasal layer consists solely of generative cells, all the labeled daughter cells that are differentiating should have migrated out from the layer by the time of one generation, i.e. by 4 days, since after one generation time all the generative cells should have divided once and increased doubly in number, thereby occupying two layers. In 4 days, however, only 66 \( m \)-cells (=1\( \times 2gm + 2\times m^2 \), cf. Fig. 7) had migrated upward out from the G-zone. This value accounts for only half of the expected number 100 (=1\( \times 50 + 2\times 25 \)). The rest of the differentiating cells should still remain in the G-zone. This finding suggests (1) that differentiating cells stay in the epibasal layer for a considerable duration of time, the average of this incubation time roughly equals 4 days, and (2) that many cells in the epibasal layer are actually differentiating cells. Since the average of the transit time of differentiating cells in the epibasal layer approximately equals the generation time of the generative cell, we may estimate that the number of differentiating cells in the epibasal layer is equal to that of the generative cells in the same layer. Suppose we label the epidermal generative cells and observe them 4 days later, half of the epibasal cells, i.e. all the generative cells in the layer should have divided once and produced the same number of daughter cells, which had admixed with the pre-existing differentiating cells in the epibasal layer. However, the number of cells in the epibasal layer is limited so that half of the differentiating cells should have transferred into the superficial layer. As we have seen in the discussion above, the process of differentiation or upward migration is a random event. Therefore, ascending cells should have been chosen at random from the newly born daughter cells and from the pre-existing differentiating cells. Thus half of the labeled differentiating cells would ascend. The expectation agrees with the actual observation. The assumption is likely that half of the cells in the epibasal layer are generative cells. Recently Frankfurt (1967) reported very similar observations in the squamous epithelium of the mouse forestomach stating that 43% of the cells in the basal layer, the only generative layer in the mouse squamous epithelium, are maturing cells. This assumption also explains why the labeling index is as low as 5.2 or 5.0% in the human epidermis whereas the duration of DNA synthesis was estimated at 7 hours (Kaku, Igarashi and Fujita 1964). In the present experiment, the labeling index was measured by counting the labeled cells in the epibasal and basal layers and dividing the number by the total number of the unlabeled and labeled cells in the epibasal layer. In this measurement, unlabeled cells in the basal layer are not included but, instead, differentiating cells in
the epibasal layer are indiscriminately taken into account. As pointed out above, generative cells in the basal layer are not so many, but non-proliferating cells in the epibasal layer amount to half as many as the total cells. If one subtracts these non-proliferating cells from the total cell count, the actual labeling index increases twice as much as the original value. Thus adjusted labeling index is $10 - 10.4\%$. Thus $L. I. = \frac{10 - 10.4}{10} = 100 - t_S/t_G$, $t_S \geq 10$ hours. This estimate of DNA synthetic period gives maximum length, since unlabeled cells in the basal layer are not taken into consideration, thus $t_S \leq 10$ hours.

**Summary**

Using *in vivo* local labeling method, cell proliferation and differentiation in the human epidermis covering the mammary gland of 41 and 42 year old women were studied by means of $^3$H-thymidine autoradiography.

1. Stratum germinativum or G-zone of the human epidermis in which cells are proliferating corresponds to epibasal and basal layers, but not the basal layer alone. Proliferating cells are predominantly located in the epibasal layer.

2. Melanin containing cell in the basal layer is a kind of minor-differentiated cell and proliferates at much slower rate than the non-pigmented basal cell.

3. Generation time of the generative cell is estimated at 4 days and the maximum length of DNA synthesis, at 10 hours. Many differentiating cells stay in the epibasal layer and they amount to 50\% of the total cells in the layer. Their transit time in the layer is estimated at an average of 4 days.

4. The unequal division or $(G, M)$ division is not a mechanism to maintain the steady state, nor do any types of mitosis play a decisive role in the regulation of cell differentiation. Mitoses yield two equivalent daughter cells which are in the same state as the mother cell.

5. Cell differentiation in the human epidermis seems to be determined by the rule of probability in an individual daughter cell during $t_1$ or $G_1$ period, as has been confirmed in various other cell systems.
ことが推定された。これらの細胞が基底層にとどまる時間は平均4日と想定される。

5. 表皮のように細胞がたえず入れかわりながら組織全体として同じ構造が保たれる定常状態は、最近まで不等細胞分裂によって維持されると考えられて来たが、この考えはオートラジオグラフ上に観察された細胞分化のパターンと合わないことがわかった。細胞レベルでの増殖と分化のバランス調節は、等分裂あるいは不等分裂といっつ細胞分裂のタイプの組みあわせにより支配されるものでなく、分裂は常に母細胞と同じ娘細胞を2つ再生すると考えるべき結果がえられた。

6. ヒトの表皮においても、他の種々の組織についてすでに報告されたと同じように、細胞分化は娘細胞が作られたのち、t1すなわちG1期の間に個々の細胞において、お互いに独立に、確率の法則に従って決定されると考えられる。

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