EFFECTS OF IRRADIATION ON THE GENERATIVE CYCLE OF THE ESTROGEN STIMULATED VAGINAL EPITHELIUM

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Abstract:
The effects of irradiation (300, 500 and 1500 rads) on mitosis and DNA synthesis in the estrogen primed vaginal epithelium have been studied. Dose—effect relations and the time sequence of effects on the two processes were investigated. The technique of tritiated thymidine labeling of DNA with autoradiography was used, in conjunction with the mitotic count, to study alterations in the generative cycle. Prior to irradiation, ovariectomized female rats were treated daily with diethylstilbestrol for a period of 2 weeks to create a steady state in the vaginal cell population.

It was observed that:

(1) Within 1 hr following ionizing radiation, mitotic figures disappear from the population and reappear at a time that is dose dependent. Those cells that have begun mitosis at the time of irradiation were able to complete that phase but no cells which were in 02 were able to begin mitosis. Therefore, a G_2 block occurs within 1 hr post-irradiation.

(2) Radiation reduces the rate of DNA synthesis thus prolonging the S phase. There is no evidence of a radiation-induced G_1 to S block in this system.

Based on these observations, it was further hypothesized that :

(1) Cells in G_1 at the time of irradiation are relatively insensitive and continue to progress through the generative cycle at a rate primarily determined by the level of estrogen stimulation.

(2) Radiation may interfere with the estrogen priming mechanism in this hormone-dependent system thereby reducing the effective level of estrogen stimulation. This is seen in the behavior of cells which were in G_1 at the time of irradiation. The extent of the blockage of estrogen increases with radiation dose and after 1500 rads, estrogen stimulation is essentially at castrate level.

The mechanisms by which ionizing radiation interferes with cellular proliferation remains controversial despite the extensive research with cell populations both *in vivo* and *in vitro* (Patt & Quastler, 1963; Dewey & Humphrey, 1965; Little, 1968). This may be due in part to the wide variation in species, tissue, dose, and time intervals used. Most cell populations are asynchronous with respect to the position of the component cells in the generative cycle. That is, at any given time an asynchronous cell population will contain cells in all stages of mitotic and interphase activity, the exact proportions of cells in any given stage being dependent upon the duration of that stage. This heterogeneity of the cell population and the variety of changes that are produced in the cells at different stages of their mitotic cycles has led to the concept that cell radiosensitivity varies according to the position of the cell in its generative cycle (Dewey & Humphrey, 1962; Sinclair & Morton, 1966; Whitmore, Gulyas & Botond, 1965). It has been established that alteration in desoxyribonucleic acid (DNA) synthesis is related to changes in cellular proliferation (Whitmore *et al.*, 1965). While the mechanisms

of radiation damage have been extensively studied using a variety of cell populations, relatively few studies have been concerned with the effects of radiation on hormone dependent systems. Our present investigation was undertaken to establish the changes in the mitotic activity and effect on DNA synthesis of ionizing radiation on the estrogen primed vaginal epithelium.

MATERIALS AND METHODS

Animals

A total of 429 female, weanling Holtzman rats were used in the study. The animals were maintained in air-conditioned quarters on standard diet. The experimental animals were divided into three groups, each receiving a total radiation dose of either 300, 500 or 1500 rads. Restraining cages were used during irradiation to prevent movement. Only the hind quarter of the rat was placed under the irradiating beam. For each experimental group, there was a corresponding control group. Control animals were kept in the restraining cages for the same length of time as the irradiated animals but were not placed under the Cesium source. The majority of the experimental groups were comprised of between four and seven animals. There was a total of 165 control animals.

Prior to irradiation all groups were bilaterally ovariectomized and received 0.05 μg diethylstilbestrol in 0.05 ml corn oil intramuscularly daily for 2 weeks to assure steady state conditions in the vaginal epithelium (Peckham & Kiekhofer, 1962).

TABLE 1. Calculated values for dose rate

Total radiation (rads)	Dose rate (rads/min)	Source–organ distance (cm)	Irradiation time (min)
1500	348·8	12·5	4·3
500	91·8	25·0	5·4
300	91·8	25·0	3·3

Irradiation

The experimental rats were irradiated with a 662 keV gamma beam from a Cesium 137 teletherapy unit. The dose rate and source-organ distances are given in Table 1. Animals were sacrificed at intervals from 0 to 72 hr after irradiation. Forty-five minutes prior to sacrifice the rats received tritiated thymidine (sp. act. 0.36 Ci/mM, Schwarz) intraperitoneally at a dose of 1 μCi/g body weight. Tritiated thymidine injection and sacrifice times for the control animals were the same as for the experimental.

At sacrifice, the vaginas were removed and processed as previously described (Peckham & Kiekhofer, 1962). Paraffin sections were cut at 3 μ and stained with the Fuelgen technique for labeling index determinations. Autoradiograms were made with the use of Kodak NTB-3 nuclear tract emulsion and were exposed for 5 weeks at - 4°C. Slides of hematoxylin and eosin stained tissues for mitotic count determinations were made from replicate sections. Both mitotic count and labeling index were determined for each animal. Labeling index was determined by a count of the number of labeled cells per 1000 basal cells. Mitotic count was determined by counting the number of mitotic figures in the basal cell layer per 100 high power fields.

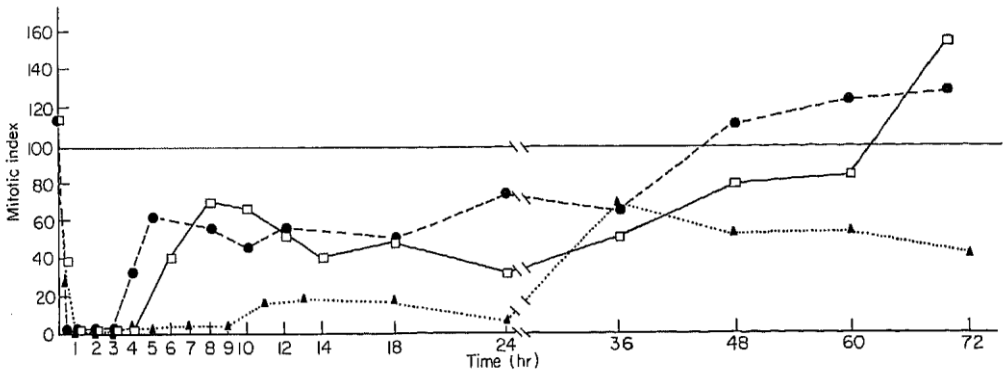

FIG. 1. Change in the mitotic index of the basalis of the estrogen stimulated rat vaginal epithelium with time after irradiation. The mitotic index is expressed as per cent of control. ●, 300 rads; □, 500 rads; ▲, 1500 rads.

RESULTS

Mitotic counts

Immediately following irradiation, the mitotic count fell precipitously and complete mitotic inhibition was reached for 300, 500 and 1500 rads (R) by 1 hr. As can be seen in Fig. 1, the length of the mitotic delay increased with increasing doses of irradiation. The release of the mitotic delay occurred at 3, 4 and 9 hr for 300, 500 and 1500 R respectively. Following the mitotic delay after 300 and 500 R, there was a rapid increase in mitotic activity followed by a sustained sub-control level which extended over a period of time (Table 2). Control levels were subsequently reached and surpassed at these lower doses of irradiation.

TABLE 2. Mean labeling index (LI) and mitotic counts (MC) after three doses of irradiation to the estrogen primed rat vaginal epithelium

Hours post-irradiation	Dose of radiation					
	300 rads		500 rads		1500 rads	
	Mean LI	Mean MC	Mean LI	Mean MC	Mean LI	Mean MC
0	—	46·7	—	47·4	—	29·8
½	—	1·0	—	15·9	—	11·3
1	15·9	0·0	17·3	0·0	18·9	0·6
2	23·0	1·5	14·4	0·5	19·5	0·0
3	20·6	1·5	15·8	0·5	22·4	0·0
4	7·2	13·2	11·7	0·4	22·1	1·0
5	7·7	26·0	—	—	13·5	1·5
6	6·8	—	9·0	17·1	—	—
7	—	—	—	—	13·0	1·8
8	6·1	23·3	5·0	29·5	—	—
9	—	—	—	—	8·3	2·0
10	17·9	19·0	4·5	27·7	—	—
11	—	—	—	—	6·6	6·7
12	9·8	23·5	9·5	21·8	5·2	7·8
14	—	—	—	16·9	—	—
18	9·0	21·0	20·5	20·0	2·0	6·7
24	25·4	30·5	13·3	13·2	4·8	2·8
36	15·6	27·0	15·9	21·0	21·3	28·7
48	25·6	45·6	8·2	32·6	12·5	21·6
60	26·5	50·7	23·4	34·2	12·1	22·0
72	17·9	52·5	29·8	62·9	13·3	16·9
Control	15·9	41·2				

After irradiation of 1500 R, the mitotic delay is evident up to 9 hr post-irradiation, although an occasional mitotic figure was observed after 4 hr. The initial rise in mitotic activity for the 1500 R group after 9 hr was much smaller than that seen in the 300 R and 500 R groups. A second rise in mitotic activity after 1500 R occurred after 24 hr. This rise, although significant, did not reach control values, and the mitotic activity of the 1500 R group remained at approximately 40 % of control for the remainder of the experiment time.

Labeling index

For labeling index studies the same animals used for mitotic count determinations were pulse labeled with tritiated thymidine at various intervals from 1 to 72 hr post-irradiation.

After 300 R (Fig. 2) there was an immediate increase in the per cent labeled cells reaching 145 % of control value by 2 hr. A sharp drop occurred after 3 hr followed by a sustained Low level of labeled cells (approximately 40 % of control) from 4 to 8 hr (Table 2). There was a second peak of labeled cells above control level at 10 hr post irradiation followed by a return to subcontrol levels at 12 and 18 hr. A third labeling index peak occurred at 24 hr, and a final prolonged peak occurred after 36 hr.

Following the dose of 500 R the mean labeling index remained at control level and there was no initial increase in per cent labeled cells. After 3 hr, a gradual decrease was seen, extending to 10 hr post-irradiation. A single wave of increased labeled cells occurred at 18 hr and a final rise above control level began after 48 hr.

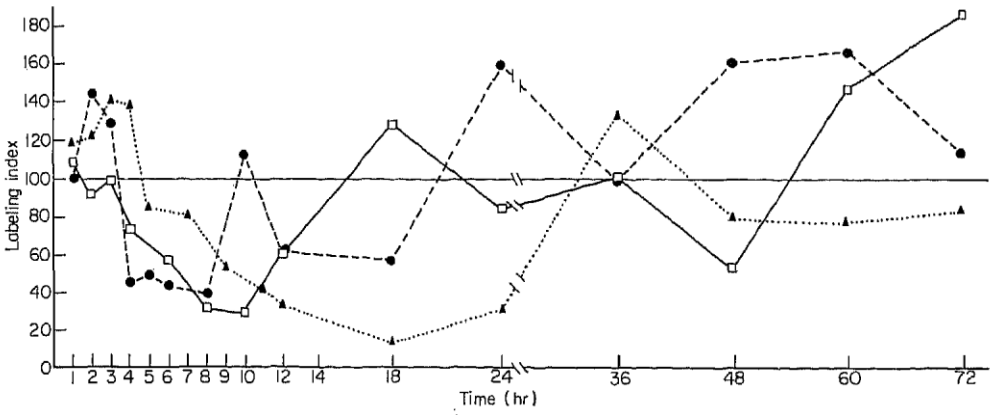

FIG. 2. Change in the labeling index of the basalis of the estrogen stimulated rat vaginal epithelium with time after irradiation. The labeling index is expressed as per cent of control. ●, 300 rads; □, 500 rads; ▲, 1500 rads.

After a total dose of 1500 R the labeling index showed an initial increase in the first several hours post-irradiation, similar to that seen for the 300 R group. After this dose, however, the increase was a more gradual one reaching its maximum at 3 hr, and was sustained for a slightly longer period than at 300 R. The subsequent decrease in labeling index was also more gradual, reaching the low point of 12% of control by 18 hr. However, by 36 hr there was an increase in synthetic activity reaching 135 % of the control value. By 48 hr, the per cent labeled cells essentially reached a plateau at approximately 80 % of control.

DISCUSSION

Effect of irradiation on mitotic count

The results of the present experiments show that immediately following ionizing radiation, mitotic figures disappear from the population and reappear at progressively later times as the dose of irradiation is increased. The duration of the mitotic delay is, therefore, apparently radiation dose dependent. This finding is well corroborated in numerous previous studies of mammalian cells both *in vivo* and *in vitro* (Elkind, Han & Valz, 1963; Kim & Evans, 1964; Watanabe & Okada, 1968; Whitmore, Till & Gulyas, 1967) yet the duration of mitotic inhibition will differ with the cell population.

It seems evident from the present study that those cells that had begun mitosis at the time of irradiation were able to complete that phase but no cells which were in G_2^* at that time were able to begin mitosis. This is shown by the fact that mitotic figures required 1 hr to completely disappear—a time equivalent to the normal mitotic time of vaginal epithelium (Peckham, Ladinsky & Kiekhofer, 1963). Therefore, a block in-this cell system induced by radiation occurs in G_2.

Following mitotic inhibition, a period of partial recovery was seen at all dose levels. After 300 R the block was released at 3 hr and the mitotic count increased rapidly to 65% of control by 5 hr post-irradiation. After 500 R the mitotic block remained 1 hr longer than at the lower dose level and the mitotic count increased more slowly reaching 70 % of control by 8 hr post-irradiation. There was no significant increase in mitotic activity above these levels until after 24 hr when the 300 R group began a gradual increase in mitotic count, By 48 hr, control levels were surpassed in this low dose group and the gradual increase continued to the end of the experimental period. The 500 R group did not show a further increase in mitotic activity until after 36 hr post-irradiation, In this middle dose group, control values of mitotic count were not exceeded until 72 hr post-irradiation.

The failure of the mitotic counts to return to control values immediately after the mitotic block was released perhaps reflects a lethally damaged cohort of the cell population and its failure to continue mitotic activity. The slower rate of increase of mitotic counts both initially and after 24 hr in the 500 R group suggests that there is a slow-down in the rate at which these cells proceed through the generative cycle.

After 1500 R the mitotic block was released by 9 hr, although there was evidence that some of the cells were able to enter M after 4 hr. The appearance of some cells in M prior to 9 hr after 1500 R may represent the slow entry into M of the cells which were in G_2 at the time of irradiation. This phenomenon may be masked at the lower dose levels because of the shorter time interval between the entry of G_2 and S cells into M after the release of the G_2 block, The rise in mitotic count after 9 hr at 1500 R would then be the entry into M of the cells in S at the time of irradiation. The reduced mitotic activity following the release of the mitotic block in the 1500 R group suggests that this highest dose of radiation lethally damaged a larger portion of the cell populations, resulting in greater loss of mitotic activity. For 1500 R the second period of increased mitotic activity occurred at 36 hr but only reached 70 % of control value followed by a drop and extended plateau at 40 % of control. This lack of complete recovery by 72 hr might further reflect the lethally damaged cohort of the cell population and its failure to continue mitotic activity. In Williams' experiments, rat intestine irradiated *in vivo* with doses of less than 700 R exhibited over-shoot (mitotic activity temporarily higher than control) and final recovery (permanent return 'of mitotic activity to control level) while doses greater than 700 R resulted in failure to recover the normal mitotic rate (Williams *et al.,* 1958). Kim & Evans (1964) also demonstrated this over-shoot phenomenon in the mitotic rate of ascitic tumor cells in culture after radiation of 1000 R.

Effect of irradiation on labeling index

The initial increase in labeling index after 300 R and 1500 R (Fig. 2) is most probably due to an irradiation-induced block in G_2 accompanied by a slow-down in the rate of DNA synthesis. After 1500 R, the G_2 block was of longer duration and the cells in synthesis were slowed down to a greater extent due to this high dose of irradiation. The fact that the extent of the G_2 block and slow-down in S is radiation dose dependent, has been amply confirmed in the literature (Dewey & Humphrey, 1965; Little, 1968). Therefore, at this higher dose level, the initial increase in number of labeled cells reached its peak more gradually than the increase after 300 R.

After 500 R, an immediate G_2 block was evident from the mitotic count data, but there was a failure of labeling index to rise above the control levels in the first 3 hr post-irradiation. This failure of the labeling index to initially rise above control values was not expected, since it implies a G_1 to S slowdown, whereas the 300 R and 1500 R labeling index data indicate that there is no radiation-induced G_1 to S block operating in this system.

After 3 hr, there was a sharp decline in per cent labeled cells to below control levels after all doses of radiation. This decline may be attributed to an increased rate of flow of cells from S to G_2 as suggested by Mak & Till (1963). The second rise in labeling index seen at 10 hr after 300 R could be explained if the population of cells in S at 2 hr had proceeded through G_2, M and G_1 and has reached the S phase again by 10 hr. This seems unlikely, however, since previous experiments dealing with the estrogen stimulated rat vaginal epithelium indicate the cycle time to be longer than 10 hr (Peckham *at al.,* 1963). If cells in the G_1 part of the cell cycle are relatively insensitive to radiation (Brent, Butler & Cathorn, 1966; Bootsma, 1964; Watanabe & Okada, 1968) and are not retarded to the same extent as cells in S and G_2, a near control value for labeling index would be expected when this G_1 population of cells enters S. This was found to be the case since at 10 hr the labeling index was not significantly different from control (112 % of control, $P > 0.5$). Therefore, this peak in labeled cells is more likely due to the cells which were in G_1 at the time of irradiation and have entered S.

Since it takes 18 hr for the second labeling index peak to appear after 500 R, a longer generative cycle time may be indicated after this dose of radiation. The fact that this labeling index peak at 18 hr was approximately 30 % higher than the mean control value may further indicate that the irradiation slowed the rate of DNA synthesis to the extent that not all of the cells in S at the time of irradiation have completed synthesis before the majority of the G_1 population was in S. Thus, a summation effect is caused by cohorts of cells which now have different generative cycle times and coincide in S resulting in a labeling index significantly above control. This summation effect was also found by Nygaard & Potter (1959) in their work on irradiated rat thymus, spleen and small intestine. After 1500 R the second labeling index peak was not evident for approximately 36 hr post-irradiation. Since this labeling index peak was nearly 60 % above the mean control value, a substantial number of cells in S at the time of irradiation would have had to remain in synthesis at 36 hr to coincide with the incoming G_1 cells.

The third rise in labeling index at 24 hr after 300 R to 159 % of control would correspond to the time when the original G_1 population of cells was in DNA synthesis for the second time, assuming a generative cycle time of roughly 15 hr. After 24 hr, interpretation of labeling index at all dose levels becomes more difficult because of increasing asynchrony due to increased heterogeneity in the length of the various phases of the generative cycle within the cell population.

Effect of estrogen on radiation response

In the present study the basal cells of the vaginal epithelium were influenced primarily by two exogenous factors: ionizing radiation and estrogen. It has been previously found that in the presence of constant estrogenic stimulation, there was an increase in labeling index with increasing dose (Peckham *at al.,* 1963). It has also been found that the generative cycle time of ovariectomized rats decreased with increasing steady state levels of estrogen stimulation, and there was a concomitant decrease in the duration of all phases of the cycle associated with the decrease in generation time (Ladinsky & Peckham, 1965).

In the present results, the second peak in labeling index, possibly corresponding to the time when the cells irradiated in G_1 were in S, occurred at 10, 18 and 36 hr after 300 R, 500 R and 1500 R, respectively. If these labeling index peaks do reflect the position of the original G_I population, this fact may be interpreted as showing an increasing generative cycle slowdown as a result of a progressive decrease in estrogen response. At the lowest dose level (300 R) the rate of progression of cells from G_1 to S was consistent with the rapid generative cycle due to the estrogen stimulation, but the rate of DNA synthesis was slowed due to the effects of irradiation. This led to more cells entering S than leaving, resulting in the increase in labeling index seen in Fig. 2.

With the progressive decrease in estrogen stimulation seen at the 500 R dose level, the labeling index remained at control level for 3 hr post-irradiation. This initial plateau at control level in the 500 R dose group indicates that the G_1 to S slow-down due to a decrease in estrogen stimulation was about equal to the rate of slow-down of cells in synthesis due to radiation resulting in a plateau of labeling index at control level.

By 1500 R, an initial increase was again seen in labeling index. If irradiation had induced a G_1 to S block after 500 R, an initial increase in labeling index would not be expected at a higher dose of radiation and the labeling index curve should be similar to that of the 500 R group. Since an initial labeling index increase was seen after 1500 R, it may be that after this high dose of irradiation the estrogen response was essentially at castrate level and the rate of DNA synthesis was reduced to such an extent that there was, again, an increased labeling index. In other words, the rate of cell entry into S from G_I after 1500 R was drastically slowed by the lack of estrogen stimulation, but the rate of cell progress through S was slowed even more drastically by the radiation resulting in the build-up of labeled cells seen at this dose level. Both the mitotic counts and the labeling indices indicate that there was a greatly increased generative cycle time after 1500 R. These facts give further support to the suggestion that the estrogen response was absent after the highest radiation dose.

The differential sensitivity of cells in different parts of the cell cycle to irradiation is well established. The present data seem to indicate that the cells in the basal layer of the vaginal epithelium are more radiosensitive in the S and G_2 phases while cells in G_1 and M are least radiosensitive. The response of the vaginal cell population to radiation may be compounded by the effect of radiation on the estrogen priming mechanism in this hormone dependent system.

When analysing data such as presented in this study, it is evident that the sampling time intervals have an important bearing on the interpretation of radiation response phenomena. Perrotta (1966) reported an 85-95 % delay of entrance of G_1 cells into S after irradiation to the mouse uterine epithelium following a single injection of estrogen. This finding was based on labeling index determinations and grain counts at a single time post-irradiation. Her technique is valid only if it is assumed that estrogen stimulates a 'resting reserve' or G_o population of cells to enter the generative cycle in G. However, Ladinsky & Peckham (1965) using continuous labeling techniques reported no evidence of a G_o population in the estrogen-stimulated rat vaginal epithelium.

The lack of a G_o population was corroborated by Galand *et al.* (1967) in the uterine and vaginal epithelium of mice. It is conceivable that results similar to the ones reported in this study would have been found for the uterine epithelium if: (1) estrogen had been made continuously available, and (2) serial sampling post-irradiation had been carried out.

In summary, it is suggested that the initial effects of irradiation on estrogen dependent tissues may be two-fold: (1) an initial G_2 block and slow-down in S with no interruption of flow of cells from G_1 to S at doses of radiation below a critical level; (2) an irradiation dose dependent interference with the estrogen priming mechanism resulting in a slow-down of the entire generative cycle as a result of decreased estrogen stimulation. This would be manifested first by a decrease in the number of cells entering S from G_1 and subsequently a decrease in the number of cells in all phases of the generative cycle. It could be that estrogen acts as a determining factor in the activation of enzymes involved in the synthesis of DNA and that irradiation interferes with the activation of such enzymes (Perrotta, 1966). If this is the case, increased doses of radiation may progressively alter the rate of activation of such enzymes.

Notes:

* G_2 = Post-synthetic, pre-mitotic phase; M = period of mitosis; G_1 = pre-synthetic, post-mitotic phase; S = period of DNA synthesis (Howard & Pelc, 1953).

REFERENCES

BOOTSMA, D. (1964) Changes induced in the first post-irradiation generation cycle of human cells studied by double labeling. *Exp. Cell Res,* 38, 429.

BRENT, T.P., BUTLER, J.A.V. & CATHORN, A.R. (1966) Effects of irradiation on synthesis of DNA and mitosis in synchronous cultures of HeLa cells, *Nature (Land.),* 210, 393.

DEWEY, W.C. & HUMPHREY, R.N. (1962) Relative radiosensitivity of different phases in the life cycle of L-P59 mouse fibroblasts and ascites tumor cells. *Radiat. Res.* 16, 503.

DEWEY, W.C. & HUMPHREY, R.N. (1965) *Cellular Radiation Biology,* p. 340. Williams & Wilkins, Baltimore. ELKIND, M.M., HAN, A. & VALZ, K.W. (1963) Radiation response of mammalian cells grown in culture. *J. nat. Cancer Inst.* 30, 705.

GALAND, P., RODESCH, F., LEROY, F. & CHRETIEN, J. (1967) Radioautographic evaluation of the estrogendependent proliferative pool in the stem cell compartment of the mouse uterine and vaginal epithelium. *Exp. Cell Res.* 48, 595.

HOWARD, A. & PELC, S.R. (1953) Synthesis of DNA in normal and irradiated cells and its relation to chromosome breakage. *Heredity,* Suppl. 6, 261.

KIM, J.H. & EVANS, T.C. (1964) Effects of X-irradiation on the mitotic cycle of Ehrlich ascites tumor cells. *Radiat. Res.* 21, 129.

LADINSKY, J.L. & PECKHAM, B.M. (1965) The kinetics of the generative compartment of the estrogen dependent vaginal epithelium. *Exp. Cell Res,* 40, 447.

LITTLE, J.B. (1968) Cellular effects of ionizing radiation. *New Engl. J. Med.* 278, 308.

MAK, S. & TILL, J.E. (1963) The effects of X-rays on the progress of L-cells through the cell cycle. *Radial. Res.* 20, 600.

NYOAARD, O.F. & POTTER, R.L. (1959) Effect of X-irradiation on DNA metabolism in various tissues of the rat. *Radiat. Res.* 10, 462.

PATT, H.M. & QUASTLER, H. (1963) Radiation effects on cell renewal and related systems. *Physiol. Rev.* 43, 357.

PECKHAM, B.M. & KIEKHOFER, W. (1962) Cellular behavior in the vaginal epithelium of estrogen-treated rats. *Obstet. Gynec.* 83, 1021.

PECKHAM, B.M., LADINSKY, J.L. & KIEKHOFER, W. (1963) An autoradiographic investigation of estrogen response mechanisms in the rat vaginal epithelium. *Amer. J. Obstet. Gynec.* 87, 710.

PERROTTA, C. (1966) Effect of X-irradiation on DNA synthesis in the uterine epithelium. *Radiat. Res.* 28, 232. SINCLAIR, W.K. & MORTON, R.A. (1966) X-ray sensitivity during the cell generation cycle of cultured Chinese hamster cells. *Radiat. Res.* 29, 450.

WATANABE, I. & OKADA, S. (1968) DNA synthesis during the first post-irradiation life cycle of lethally irradiated cultured mammalian cells (L5178Y). *Radiat. Res.* 35, 202.
WHITMORE, G.F., GULYAS, S. & BOTOND, J. (1965) In : *Cellular Radiation Biology,* p. 423. Williams & Wilkins, Baltimore.
WHITMORE, G.F., TILL, J.E. & GULYAS, S. (1967) Radiation-induced mitotic delay in L-cells. *Radial. Res,* 30, 155.
WILLIAMS, R.B., TOAL, J.N., WHITE, J. & CARPENTER, RN. (1958) Effect of total-body X-radiation from near-threshold to tissue lethal doses on small-bowel epithelium of the rat. *J. nat. Cancer Inst.* 21, *17.*