The Effects of Diethylstilbestrol (DES) on the Cell Cycle of DES-induced Renal Carcinoma of Syrian Hamster in vitro

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INTRODUCTION

A hormonal regulation of the cell cycle has been studied by many investigators[1-11] and Epifanova[12] demonstrated that estrogen accelerates the cell cycle of the target tissue. However, the effects on non-target tissues remain to be elucidated. Although the kidney is primarily a non-target tissue of estrogen, it was shown by Kirkman et al[13-17] that an estrogen-dependent kidney tumor can be induced by repeated injection of diethylstilbestrol (DES) a synthetic estrogen to male Syrian hamster. The present investigation was motivated by a successful culture in vitro[18] of the DES-induced renal carcinoma of Syrian hamster which has been serially transplanted in ascites form in DES-treated animals. As will be detailed elsewhere[18], the growth of the culture has been maintained in the medium without estrogen.

This paper describes cell-kinetic aspects of hormone actions on the tumor using this culture line. The use of cell culture has definite advantage over the in vivo experiment because indirect effects which are manifested through endocrine glands of host animals can be excluded. However, a longcontinued growth of cells under the influence of hormone leads to acclimatization with resultant biochemical, cytological, or genetic changes in the cells of object due to induction, selection, or mutation. On the other hand, a sudden transfer of the culture to a new culture medium containing hormone will perturb the cell population and will generate a transient state to which none of the present day methods[19-21] of cell cycle analysis is applicable. In this investigation, an approach was made to the problem by resolving the latter difficulty using a recently developed computer program for the transient state FLM curve analysis[26] which is a modification of Takahashi-Hogg-Mendelsohn program[22]. Also will be discussed the mode of cell loss under the influence of high dosis of DES.

MATERIALS AND METHODS

Using cell culture of DES-induced renal carcinoma, four series of experiment with four different concentrations of DES in the medium were performed and the data of the fraction of labeled mitoses, population doubling time, and labeling index were obtained.

Cell culture: The cell was derived from the DES dependent renal carcinoma which was induced in male Syrian hamster by repeated subcutaneous injection of diethylstilbestrol and was transformed to ascites tumor. The ascites tumor had been transplanted for 40 generations before it was cultured in vitro. The nutrient medium was Ham's F12[23] supplemented with 10% calf serum and 0.06 mg/l Cephamedine but without addition of any hormone. The cells formed monolayer in TD40 flask. Subcultures were made at about 7-day interval by trypsinization and the cells were in an almost exponential growth phase most of the time. The following experiments were performed after maintenance of the culture for 640 days.

H^3-thymidine containing medium: Ham's F12 deprived of thymidine was prepared and H^3-thymidine (The Radiochemical Center, Amersham, specific activity 2.0 Ci/mM) was added to a final concentration of 0.25 μCi/ml.

DES containing medium: Dipotassium-diethylstilbestrol-disulfate (DES-dps) (Hokuriku Pharmaceutical Co., Ltd.) a water soluble derivative of DES was used. However, a word DES will be used for DES-dps for ease of description. Media with four different DES concentrations were prepared: (1) DES free (control), (2) 3.79 mgDES/dl, (3) 7.58 mgDES/dl, (4) 11.37 mgDES/dl.

Plating on slide glass: When cells were spread entirely over the surface of TD40 flask, they were harvested by scraping with rubber policeman. In each series of experiment, cells from 8 flasks were evenly plated on 16 plastic Petri dishes (ø 10 cm) in which two sheets of slide glass were laid. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and on 5 th day the cultures were used for experiment: one for measurement of population doubling time and 15 other dishes for the fraction of labeled mitoses (FLM).

FLM experiment: Cultures were pulse-labeled in H^3-thymidine medium for 20 min and washed twice with pre-warmed (−) PBS. Then, the slides were transferred into DES medium. After variable time intervals the slides were taken out in succession and fixed in ethanol-acetic acid (3:1). The total time of sampling after pulse labeling was extended to about

50 hours. After 12 hour fixation, the slides were rinsed in running water. For autoradiography, the slides were dipped into liquid emulsion (Type ET2F, Fuji Photo Film Co., Ltd.). The coated slides were exposed for 7 days at 4°C in a refrigerator. The autoradiographs were developed and stained with hematoxylin and eosin. For the construction of FLM curves, at least 100 mitoses were observed in each sample and those having more than 5 grains were scored as labeled mitoses.

Labeling index: The ratio of labeled cells to total cell population was also measured from a score of at least 10,000 cells.

Doubling time: Population doubling time was determined in exponential growth phase by cell counts performed at intervals of 0, 24, 44, and 72 hours. Slides on each of which 8 circles of approximately 0.5 cm in diameter were marked with glass pen were used for this purpose. Total number of cells that are present within these circles were counted by an inverted microscope.

Analysis of FLM data: Two different versions of computer program were used; the one is Takahashi-Hogg-Mendelsohn program which is for a steady state population and the other is for a transient state of growth. Four data sets of FLM's for different DES concentrations were analyzed using both programs to see how important is the use of appropriate program.

RESULTS

Cell cycle: The FLM data from experiments were shown in Table 1. The extracted parameters of the cell-cycle are shown in Table 2. The parameters extracted using previous program are in the parenthesis. The generation time T_C obtained by the new program was almost equal to T_C calculated by the previous program but the new program remarkably shortened the length of G1 phase when compared to the previous program. In contrast, T_S and T_{G_2} were slightly over the values obtained by previous program. Figures 1-4 illustrate the data points and fitted curves where the confidence interval due to binomial error of sampling was indicated by a vertical line through the data point and each curve is the best fit based on the computer analysis. The solid line is the curve that was fitted using transient-state oriented method and dotted line was based on the previous program.

Both curves fitted by different programs run very closely although the parameters associated with them were remarkably different. When DES was added in concentrations of 7.58 mg/dl and 11.37 mg/dl, the data were

Table 1. Fraction of labeled mitoses

	DES-free				3.79 mgDES/dℓ				7.58 mgDES/dℓ				11.37 mgDES/dℓ			
No.	t	LM	M	FLM	t	LM	M	FLM	t	LM	M	FLM	t	LM	M	FLM
1	1.0	2	80	0.03	1.0	3	100	0.03	1.0	3	102	0.03	1.0	5	101	0.05
2	2.0	4	81	0.05	2.0	10	100	0.10	2.0	12	100	0.12	2.0	4	100	0.04
3	3.0	21	80	0.26	3.0	10	100	0.10	3.0	7	100	0.07	3.0	5	100	0.05
4	4.0	17	59	0.29	4.0	26	100	0.26	4.0	11	101	0.11	6.0	16	102	0.16
5	5.0	54	100	0.54	5.0	25	103	0.24	5.0	27	102	0.26	7.0	32	101	0.32
6	6.0	62	100	0.62	6.0	55	102	0.54	6.0	44	103	0.43	8.0	62	100	0.62
7	7.0	67	101	0.66	7.0	69	100	0.69	7.0	62	100	0.62	9.0	72	100	0.72
8	8.0	79	100	0.79	8.1	71	100	0.71	8.0	76	101	0.75	10.0	87	101	0.86
9	9.0	72	100	0.72	9.1	85	100	0.85	9.0	78	100	0.78	11.0	76	100	0.76
10	10.0	96	110	0.87	10.0	86	100	0.86	10.0	97	100	0.97	12.0	60	100	0.60
11	11.0	73	100	0.73	11.1	105	110	0.95	11.0	66	100	0.66	13.0	57	100	0.57
12	12.0	73	100	0.73	12.0	73	103	0.71	12.0	68	100	0.68	14.0	55	101	0.54
13	13.0	133	204	0.65	13.0	33	101	0.33	13.0	21	102	0.21	16.0	34	103	0.33
14	14.0	102	200	0.51	14.0	56	100	0.56	14.0	50	104	0.48	18.0	20	102	0.20
15	15.0	107	154	0.69	16.0	47	100	0.47	16.0	11	102	0.11	20.0	42	100	0.42
16	16.0	83	152	0.58	18.0	26	100	0.26	18.5	32	106	0.30	22.0	26	102	0.25
17	18.0	34	100	0.34	20.0	35	203	0.17	20.0	50	100	0.50	24.0	22	104	0.21
18	20.0	20	108	0.19	22.1	26	100	0.26	22.0	35	102	0.34	26.0	70	100	0.70
19	22.0	40	112	0.36	24.0	29	100	0.29	24.0	48	100	0.48	28.0	69	101	0.68
20	24.0	31	100	0.31	27.1	40	104	0.38	26.0	36	101	0.36	30.0	48	100	0.48
21	28.0	29	104	0.28	28.2	32	100	0.32	28.0	27	100	0.27	32.0	31	100	0.31
22	30.0	36	111	0.32	30.2	45	101	0.45	30.0	36	100	0.36	38.0	22	100	0.22
23	24.0	39	100	0.39	32.0	34	100	0.34	32.0	16	100	0.16	41.0	16	100	0.16
24	36.0	59	113	0.52	34.0	56	100	0.56	33.0	5	76	0.07	44.0	20	102	0.20
25	40.0	48	109	0.44	36.0	47	101	0.47	36.5	31	100	0.31	47.0	22	100	0.22
26	46.0	46	102	0.45	38.0	33	103	0.32	41.0	6	50	0.12				
27	48.0	39	102	0.38	40.0	40	100	0.40	44.0	42	100	0.42				
28					42.0	47	102	0.46	46.0	53	100	0.53				
29					44.0	63	212	0.30								
30					46.0	43	101	0.43								

scattered considerably. A systhematic deviation of the curve from the data around the second and third waves was noted and this deviation was increased with rising DES concentrations. In the neighborhood of the second wave the actual data tended to precede the synthetic curve while the synthetic third wave was above the data points. Biological significance of these deviations will be discussed later.

Mean cycle time T_C was remarkably reduced with increasing DES concentration. The phase that contributed most to this reduction was G1 phase and T_{G_1} was shortest with 7.58 mgDES/dl. With higher dosis of DES, T_S was also decreased, but to the contrary, T_{G_2} was increased.

Table 2. Cell cycle parameters

DES	T_{G_1}	T_S	T_{G_2}	T_M	T_C
Control	12.871 (12.337)	11.881 (12.766)	5.435 (5.035)	0.832 (0.868)	31.019 (31.007)
3.79 mg/dl	4.618 (8.735)	12.697 (9.550)	5.543 (5.691)	1.392 (0.892)	24.187 (24.869)
7.58 mg/dl	0.538 (4.339)	7.880 (6.005)	7.058 (6.346)	0.997 (0.626)	16.453 (17.315)
11.37 mg/dl	1.411 (3.224)	8.301 (6.558)	8.497 (7.612)	1.167 (0.902)	19.378 (18.296)

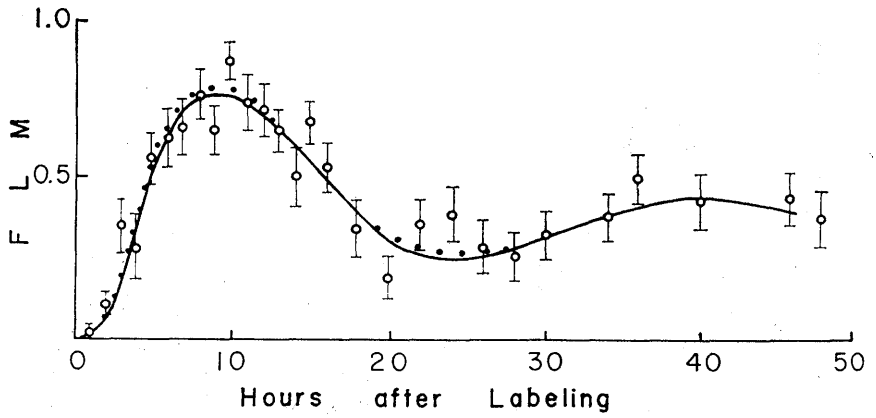

Fig. 1. FLM Analysis of Control Culture

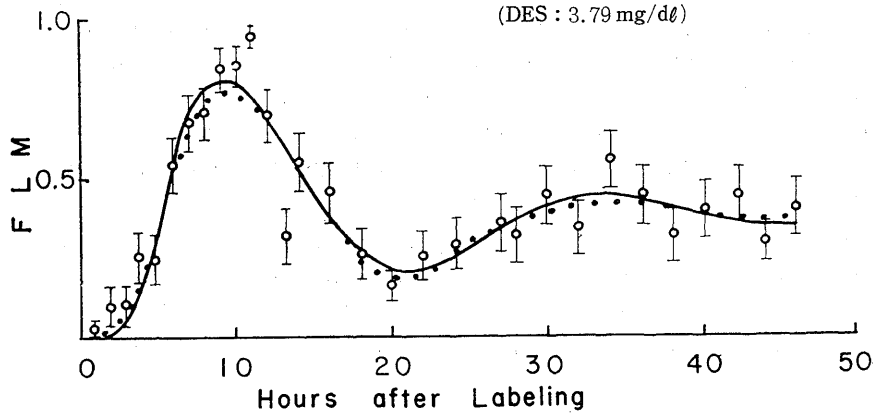

Fig. 2. FLM Analysis of DES-Stimulated Culture

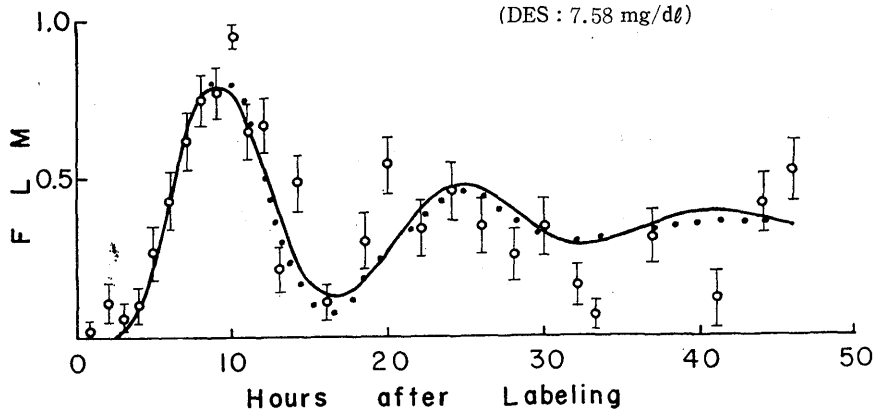

Fig. 3. FLM Analysis of DES-Stimulated Culture

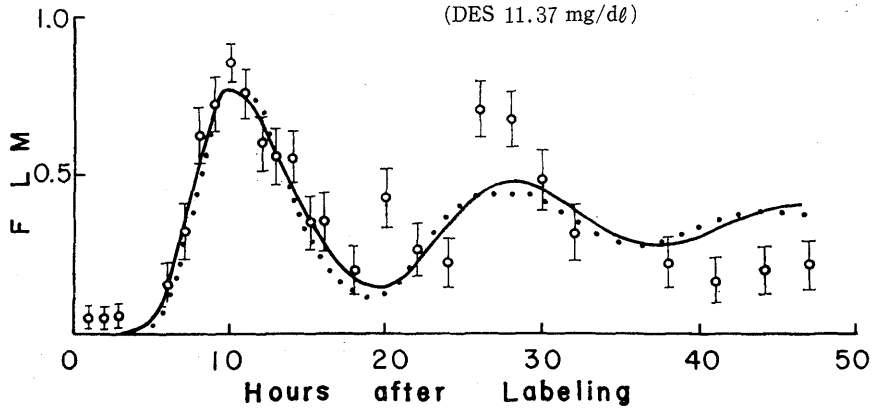

Fig. 4. FLM Analysis of DES-Stimulated Culture

Doubling time: Growth of cultures in various concentrations of DES are summarized in Table 3. The figure in each parenthesis is the ratio of cell number at time t, N(t) to the initial cell number N(0). Using a relation

$$N(t) = N(0)e^{bt},$$

the growth rate b was calculated for each t and b's at different t's were averaged for each set of experiment. The actual doubling time Td was calculated by

$$T_d = \frac{\ln 2}{b}$$

As shown in Table 4, the doubling times were slightly less than that of control culture but their relation to DES concentration was not so clear as the phase lengths.

Table 3. Growth of cell population

DES	0 hr	24 hr	44 hr	72 hr
Control	1671 (1.00)	2410 (1.44)	3332 (1.99)	4293 (2.57)
3.79 mg/dl	3310 (1.00)	5267 (1.59)	7401 (2.24)	10301 (3.11)
7.58 mg/dl	2702 (1.00)	4472 (1.67)	5849 (2.16)	7372 (2.73)
11.37 mg/dl	3048 (1.00)	4923 (1.62)	6759 (2.22)	9976 (3.27)

Cell loss: The cell loss factor ϕ was calculated according to Steel's method[24]

$$\phi = 1 - \frac{T_d^{pot}}{T_d}$$

where T_d^{pot} is the potential doubling time. The potential doubling time was calculated by

$$T_d^{pot} = \lambda \frac{T_s}{LI}$$

and putting this into

$$\lambda = \frac{T}{T_s}\left\{\exp\left[\frac{\ln 2}{T}(T_{G_2}+T_s)\right] - \exp\left[\frac{\ln 2}{T}T_{G_2}\right]\right\}$$

λ is renewed. The process was iterated until T was stabilized. With a certain reservation T converges to T_d^{pot}. The results of calculation were included in Table 4.

Table 4. Labeling index, Doubling time, Cell loss factor ϕ and Growth fraction

DES	L1	Td	Td ^{ot}	ϕ	G.F.
Control	0.249	47.208	42.761	0.094	0.606
3.79 mg/dl	0.249	38.800	42.842	−0.104	0.475
7.58 mg/dl	0.249	39.619	28.622	0.278	0.521
11.37 mg/dl	0.249	38.124	30.728	0.194	0.582

Growth fraction: Growth fraction (G. F.) was calculated by Mendelsohn's method[25]

$$\frac{\text{Labeled cells}}{\text{100 cells}} \Big/ \frac{\text{Labeled mitoses}}{\text{100 mitoses}} = \text{Growth fraction}$$

where $\frac{\text{Labeled cells}}{\text{100 cells}}$ is the labeling index and $\frac{\text{Labeled mitoses}}{\text{100 mitoses}}$ is approximated by $\frac{T_S}{T_C}$. As shown in Table 4, the growth fractions increased with rising DES concentration but was not over the value of control.

DISCUSSION

A quantitative analysis on direct effects of drugs, hormones, radiations or other agents to the cell cycle is difficult, primarily because current methods of data analysis is not applicable to a transient state of growth such that follows a perturbation of the cellular system by these agents. The outstanding point of the present investigation is a challenge against this problem by the use of a transient-state-oriented method of FLM analysis.

A culture that has been propagated continuously in an estrogen-free medium may be regarded as being in a steady state. By the addition of hormone, however, the cell population is shift towards a new steady state so that the population is in a transient state intermediate between the two steady states. Prerequisite to this analysis is the extraction of cell-cycle parameters from the estrogen-free culture. This is not only to obtain control data but to initialize the transient state FLM analysis by determining age distribution before treatment. Although the details will be described elsewhere[26], the program synthesizes a transient state FLM curve starting with the steady state age distribution and progressing with new parameters and then optimizes the parameters by way of a search for

the best-fitting FLM curve with iteratively changing parameters.

A configuration of the transient state FLM curve is by no means different from the curve for the steady state population and the same FLM data are fitted equally well by either model. However, the parameters associated with it may be different depending upon which one of the model is adopted. The present investigation has demonstrated that it was actually the case and that a choice of the appropriate program is essential.

Also important is the fact that the second wave of the experimental FLM curves seems to precede the synthetic wave and the degree of precedence increases with rising DES concentration. Despite of a local deviation from the data set, a global fit of the synthetic curve should be the best conceivable within a range of freedom of the model. Should T_{G1} be reduced to bring the second wave to the left, T_C is decreased accordingly and the third wave or the plateau which is roughly representing T_S/T_C runs high above the data. Furthermore, in order to fit the second wave to the data with 7.58 mgDES/dl, it is not enough to reduce T_{G1} to zero. A compromise to this situation is to incorporate into the model a couple of new assumptions such as mobilization of Q-cells into the cycle or a switch to semiconservative mode of DNA segregation under the influence of DES.

The cell cycle parameters computed from the FLM curves indicate that the addition of DES to the culture causes decrease of T_C to about 50% of the control, and that the phase which contributed most to this reduction was G1 phase. T_S was also decreased considerably, but to the contrary, T_{G2} was slightly increased with higher concentration of DES. The population doubling time T_d of DES-stimulated culture was approximately 20% less than the control. This discrepancy between the reductions of T_C and of T_d can be accounted for either by the increase of cell loss and by the decrease of growth fraction. The cell loss factor ϕ was minimum with low concentration of DES but was increased with higher concentration. It is probably because DES is both stimulative as well as toxic and with a large dosis a toxicity overwhelmes the cycle-stimulating effect. A decrease of the growth fractions by the addition of DES may be explained that P cells are more sensitive than Q cells to the toxic effect of DES. A fundamental problem involved in this explanation is how to calculate the cell loss factor and the growth fraction in transient state of growth, because the current methods can only be applied legitimately to steady state population. A solution to this must await further theoretical study.

Stimulatory effect of estrogens on the cell cycle in castrated female mice, using uterine epithelium as a target tissue and corneal epithelium as

a nonspecific tissue, was studied by Epifanova[12]. She found that estrone reduced the generation time of the uterine epithelial cells from 42 hr (in untreated, castrated mice) to 26 hr (in treated, castrated mice) at the expense of the G1 and S periods. There was also a 3-fold increase in the number of cells synthesizing DNA when compared to the untreated, castrated animal. In contrast, corneal epithelium of estrone-treated mice showed no essential change in the FLM curve and only a slight increase in labeling index was noted. Using vaginal epithelium of adult mice, Thrasher et al[27] demonstrated that estrogen decreased the mean cycle time from maximum of 72 hr (diestrus) to approximately 25 hr (estrus) due to a reduction in T_{G_1} while S phase (about 7.5 hr) remained unchanged. Bresciani[28], working with the mammary glands of C3H mice, noted that the labeling index and T_S varied greatly depending on temporal relationship between the time of experiment and the period in estrus cycle. But, ovariectomy plus daily supplement with progesterone reduced the T_S to about 50% of the control and he concluded that ovarian hormones act on the size of the proliferative population and modified the T_S of normal mouse mammary glands. Weihs and his associates[9] found a 50% decrease in the duration of DNA synthesis in the cells of the mammary glands and uterus of castrated estrogen-supplemented C3H mice. Simpson-Herren et al[30] showed that the DMBA-induced rat mammary tumor, regressed by ovariectomy and subsequently stimulated by estradiol and progesterone, had a T_C about 20% longer than that found for the induced tumor in intact hosts but had a 2-fold higher labeling index and shorter T_d. Combs et al[31] reported, however, that the FLM data of DMBA-induced rat mammary tumors collected at different stages of estrus were quite similar and they considered that the cell-cycle was unchanged by physiological fluctuations of hormone level during estrus cycle.

In vitro experiment is more useful for the study of direct effects of hormone. Siegel and Tobias[32], using a heteroploid cell line of human kidney cells ("T" cells) cultured in various concentration of thyroxine, demonstrated that the growth was accelerated, the plating efficiency increased and uptake of tritiated uridine stimulated. Working with the same experimental system, Burki and Tobias[33] reported also that the generation time was reduced by thyroxine from 27 hr to 20 hr due to acceleration of G1 period while dead cell fraction remained unchanged.

Despite some discrepancy of reported data, the results are basically in accord with those obtained in this experiment. Noteworthy in the present results is the fact that this DES-induced renal carcinoma has retained a sensitivity to estrogen and grows faster in response to DES, even after long sojourn in vitro without addition of DES.

Not only does this report add some information to the literature on the cell cycle but lays the data analysis on a sound basis. Future work along this line will find a vast field of application of this analytic method.

SUMMARY

The effects of diethylstilbestrol (DES) on the cell cycle of culture of DES induced renal carcinoma of Syrian hamster was studied using a recently developed computer program for a transient state FLM curve analysis. It was demonstrated that T_C was reduced to about 50% due to reduction of T_{G1} and T_S even with such high dosis of DES that the cell loss was rather increased.

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REFERENCES

1) Peckham, B. and Kiekhofer, W.: Cellular behavior in the vaginal epithelium of estrogen-treated rats. *Am. J. Obst. Gynec.*, 83 : 1021-1027, 1962.
2) Bullough, W. S. and Laurence, E. B.: Duration of epidermal mitosis in vitro. Effect of the chalone-adrenalin complex and of energy production. *Exp. Cell Res.*, 35 : 629-641, 1964.
3) Puck, T. T., Sanders, P. and Petersen, D.: Life cycle analysis of mammalian cells. II. Cells from the chinese hamster ovary grown in suspension culture. *Biophys. J.*, 4 : 441-450, 1964.
4) Stockdale, F. E. and Topper, Y. J.: The role of DNA synthesis and mitosis in hormone-dependent differentiation. *Proc. Nat. Acad. Sci. U. S.*, 56 : 1283-1289, 1966.
5) Galand, P., Rodesch, F., Leroy, F. and Chretien, J.: Radioautographic evaluation of the estrogen-dependent proliferative pool in the stem cell compartment of the mouse uterine and vaginal epithelia. *Exp. Cell Res.*, 48 : 595-604, 1967.
6) Beato, M., Lederer, B., Boquoi, E. and Sandritter, W.: Effect of estrogens and gestagens on the initiation of DNA synthesis in the genital tract of ovariectomized mice. *Exp. Cell Res.*, 52 : 173-179, 1968.
7) Frankfurt, O. S.: Effect of hydrocortisone, adrenalin and actinomycin D on transition of cells to the DNA synthesis phase. *Exp. Cell Res.*, 52 : 220-232, 1968.
8) Martin, L. and Finn, C. A.: Hormonal regulation of cell division in epithelial and connective tissues of the mouse uterus. *J. Endocr.*, 41 : 363-371, 1968.
9) Turkington, R. W.: Hormonal dependence of DNA synthesis in mammary carcinoma cells in vitro. *Science*, 160 : 1457-1459, 1968.
10) Turkington, R. W.: Hormone-induced synthesis of DNA by mammary gland in vitro. *Endocr.*, 82 : 540-546, 1968.
11) Turkington, R. W. and Ward, O. T.: DNA polymerase and DNA synthesis in mammary carcinoma cells. *Biochim. Biophys. Acta*, 174 : 282-290, 1969.
12) Epifanova, O. I.: Mitotic cycle in estrogen-treated mice: a radioautographic study. *Exp. Cell Res.*, 42 : 562-577, 1966.

13) Kirkman, H. and Bacon, R. L.: Estrogen-induced tumors of the kidney. I. Incidence of renal tumors in intact and gonadectomized male golden hamsters treated with diethylstilbestrol. *J. Nat. Cancer Inst.*, 13 : 745-755, 1952.
14) Kirkman, H.: Estrogen-induced tumors of the kidney. IV. Incidence in female Syrian hamsters. *Nat. Cancer Inst. Monogr.*, 1 : 59-92, 1959.
15) Kirkman, H. and Bacon, R. L.: Estrogen-induced tumors of the kidney. II. Effect of dose, administration, type of estrogen, and age on the induction of renal tumors in intact male golden hamsters *J. Nat. Cancer Inst.*, 13 : 757-771, 1952.
16) Kirkman, H.: Estrogen-induced tumors of the kidney. III. Growth characteristics in the Syrian hamster. *Nat. Cancer Inst. Monogr.*, 1 : 1-58, 1959.
17) Kirkman, H. and Robbins, M.: Estrogen-induced tumors of the kidney. V. Histology and histogenesis in the Syrian hamster. *Nat. Cancer Inst. Monogr.*, 1 : 93-140, 1959.
18) Nishimura, H., Masuda, M. and Takahashi, M.: The establishment of a culture cell line from DES-induced renal carcinoma of Syrian hamster. (in prep.)
19) Lennartz, K. J. und Maurer, W.: Autoradiographische Bestimmung der Dauer der DNS-Verdopplung und der Generationszeit beim Ehrlich-Ascitestumor der Maus durch Doppelmarkierung mit ^{14}C- und ^{3}H-Thymidin. *Z. Zellforsch.*, 63 : 478-495, 1964.
20) Barrett, J. C.: A mathematical model of the mitotic cycle and its application to the interpretation of percentage labeled mitoses data. *J. Nat. Cancer Inst.*, 37 : 443-450, 1966.
21) Takahashi, M.: Theoretical basis for cell cycle analysis. II. Further studies on labeled mitosis wave method. *J. Theoret. Biol.*, 18 : 195-209, 1968.
22) Takahashi, M., Hogg, J. D. and Mendelsohn, M. L.: The automatic analysis of FLM curves. *Cell Tissue Kinet.*, 4 : 505-518, 1971.
23) Ham, R. G.: Clonal growth of mammalian cells in a chemically defined, synthetic medium. *Proc. Nat. Acad. Sci. U. S.*, 53 : 288-293.
24) Steel, G. G.: Cell loss from experimental tumours. *Cell Tissue Kinet.*, 1 : 193-207, 1968.
25) Mendelsohn, M. L.: Proliferation in spontaneous breast cancer of the C3H mouse. III. The growth fraction. *J. Nat. Cancer Inst.*, 28 : 1015-1029, 1962.
26) Takahashi, M.: FLM curve analysis in transient state of growth. (in prep.)
27) Thrasher, J. D., Clark, F. I. and Clarke, D. R.: Changes in the vaginal epithelial cell cycle in relation to events of the estrus cycle. *Exp. Cell Res.*, 45 : 232-236, 1966.
28) Bresciani, F.: Effect of ovarian hormones on the duration of DNA synthesis in cells of the C3H mouse mammary gland. *Exp. Cell Res.*, 38 : 13-32, 1965.
29) Weihs, S. E. D., Truhlsen, W. C. and Banerjee, M. R.: Further evidence for hormonal influence on the duration of DNA synthesis. *J. Cell Biol.*, 43 : 155a-156a, 1969.
30) Sympson-Herren, L. and Griswold, D. P. Jr.: Studies of the cell population kinetics of induced and transplanted mammary adenocarcinoma in rats. *Cancer Res.*, 33 : 2415-2424, 1973.
31) Combs, J. W., Mackey M. and Bennington, J. L.: Regulation of cell proliferation in DMBA-induced rat mammary neoplasms by the estrus cycle. *Am. J. Path.*, 62 : 61a-62a, 1971.
32) Siegel, E. and Tobias, C. A.: Action of thyroid hormones on cultured human cells. *Nature*, 212 : 1318-1321, 1966.
33) Burki, H. J. and Tobias, C. A.: Effect of thyroxine on the cell generation cycle parameters of cultured human cells. *Exp. Cell Res.*, 60 : 445-448, 1970.