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Evidence of a G_1 Regulation of Circadian Proliferation in Mouse Methylcholanthrene-Induced Sarcomas

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Abstract—*In two methylcholanthrene induced sarcomas of C57BL male mice presenting a circadian proliferation (peak of mitoses at noon and peak of labelling indices between midnight and 4 a.m.), the authors performed per cent labelled mitosis curves every* 6th hr *during the* 8th *diurnal period of growth. The G_1 phase showed the most important variation with a significant increase in the groups receiving ^{3}H-thymidine at 2 a.m. The possibility that this periodic variation of G_1 phase regulates the circadian rhythm of cell proliferation is discussed.*

INTRODUCTION

A CIRCADIAN variation of cell division has been described, not only in rapidly proliferating normal tissues [1–23], but also in non-proliferating tissues triggered into active regeneration [24, 25]. A few papers also mention the existence of a circadian rhythm of cell division in animal [2, 26–29] and in human tumours [30–36].

In a preliminary report, we described significant circadian waves in the mitotic indices and labelling indices after injection of tritiated thymidine (^{3}H-TdR) in methylcholanthrene (MCA) induced sarcomas of mice [28]. In the present work, we have tried to extend the previous observations by comparing the circadian variations in two experimental sarcomas with different growth rates. Besides the changes of labelling and mitotic indices, the duration of the cell cycle was studied by the per cent labelled mitosis (PLM) method during a nyctohemeral period in order to approach the mechanism of nyctohemeral wave regulation.

MATERIALS AND METHODS

Two sarcomas, T_9 and T_{10}, were induced in 1969 by implanting MCA pellets in C57BL male mice; each month, the tumours are serially transplanted by trocar under the skin of isogeneic animals.

The growth curves were obtained by calculating the mean individual diameter and then the mean log volume in 30 animals during the whole period of tumour growth. The mean individual diameter (mD) was obtained by the formula: $mD = \sqrt[3]{(d^2D)}$ where d and D are respectively the minor and the major diameters of the tumour. The mean log volume and standard error of this mean were expressed for every step of tumoral growth and plotted against time on a semi-log scale.

The variations of the mitotic indices (M.I.) and of the labelling indices (L.I.) were studied during the 8th diurnal period following grafting. Five animals were sacrificed every 4th hour. One hour prior to sacrifice, 1 μCi/g of ^{3}H-TdR (specific activity: 5 Ci/mM, Radiochemical Center, Amersham) was injected in 1 ml of saline (i.p.). Tumours were resected, formalin-fixed and treated by classical histological and autoradiographical methods (Ilford L_4 emulsion, 3 week exposure, Carazzi–hematoxylin staining). To obtain a representative pattern of the whole cell population, sections (4 μm) were cut at four different levels of the tumour. The M.I. and L.I. were calculated after scoring 7000–10,000 cells per tumour; a nucleus with three grains or more was considered as labelled. The Student t-test was used to compare midday and midnight values.

To estimate the duration of the various phases of the cell cycle, the PLM method (37–39) was used. The animals were pulse-labelled either at 2 a.m., 8 a.m., 2 p.m. or 8 p.m. by i.p. injection with 1 μCi/g in 1 ml of

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tritiated thymidine. Three to five animals of each group were sacrificed at 3 hr intervals throughout a 27-hr period. A minimum of 100 mitotic figures per animal were scored either as labelled (three or more grains per figure) or unlabelled.

The durations of the cell cycle (Tc) and of the cell cycle phases ($TG_2+M/2-TS-TG_1+M/2$) were estimated by the graphical Quastler–Sherman method of the 50% intercept [37]; if the last experimental point did not reach the 50% of labelled mitoses, an extrapolation was made to this intercept. The Student *t*-test was used to compare values obtained at the 27th hr for each tumour.

RESULTS

Both tumours showed a gompertzian type of growth (Fig. 1), T_9 growing slower than T_{10}.

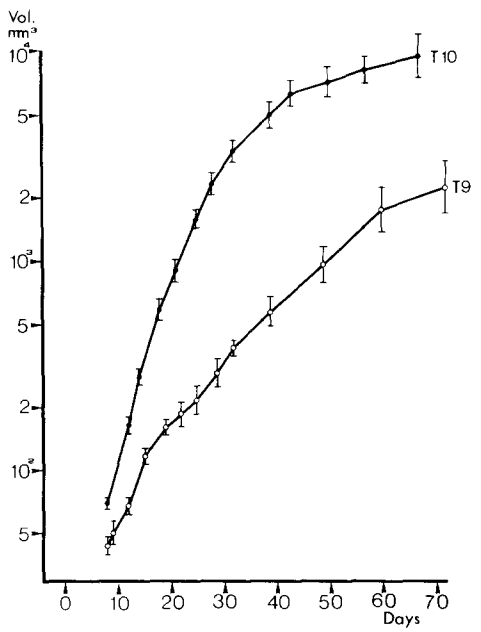

Fig. 1. Tumoral growth curves: evolution of the mean log volume and standard error of this mean along time.

The variations of mitotic and labelling indices observed during the 8th diurnal period of growth were qualitatively identical in T_9 and T_{10} (Fig. 2). The mitotic peaks were found at midday and corresponded to minimum labelling indices. On the contrary, the highest labelling indices were encountered at midnight (T_9) or between midnight and 4 a.m. (T_{10}) at a moment where mitotic indices were the lowest.

The analysis of PLM curves drawn every 6th hr at day 8 (Figs. 3a and b, 4a and b, Table 1) indicate that, in spite of some diurnal fluctuation of G_2 and S phases, the G_1 period shows the most significant variation in

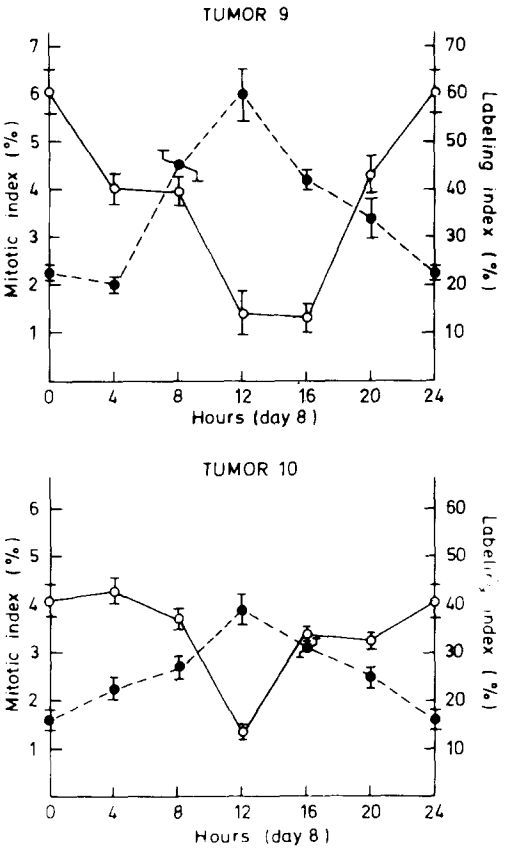

Fig. 2. Variation of mitotic indices (dotted lines) and ^{3}H-TdR labelling indices (full lines) in T_9 and T_{10} along the 8th diurnal period ($P<0.001$ between 12 a.m. and 12 p.m. means). Each point represents the mean of 5 measurements; vertical bars the standard error of this mean.

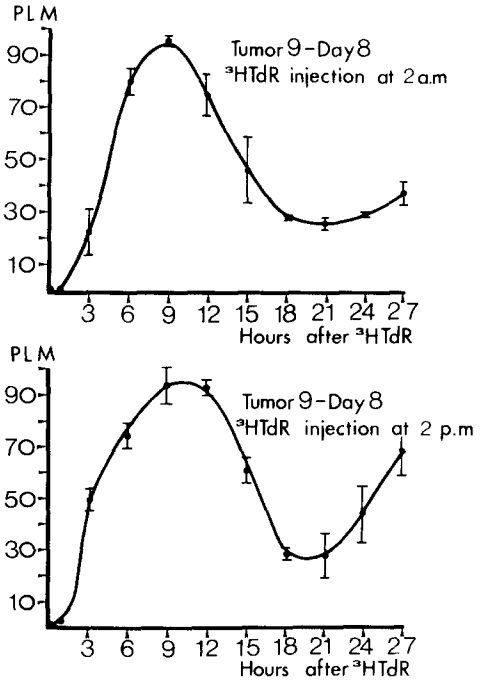

Fig. 3a.

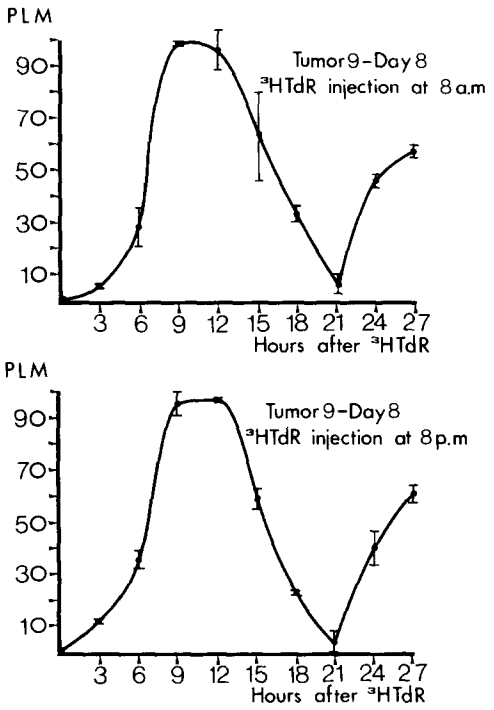

Fig. 3b.

Fig. 3. Evolution of the PLM curve in T_9 *at* day 8 *after a* ^{3}H-*TdR injection performed at* 2 a.m., 2 p.m. (*Fig. 3a*), 8 a.m. *and* 8 p.m. (*Fig. 3b*). *Each point represents the mean of* 3–5 *measurements; vertical bars the standard error of this mean.*

both tumours: the tumours labelled with ^{3}H-TdR at 2 a.m. had an important increase of their G_1 period. The lengthening of the G_1 period is confirmed: (1) by the clearcut reascension of the PLM curves obtained in both tumours after ^{3}H-TdR injections at 2 p.m., 8 a.m. and 8 p.m. contrasting with the flattening of the curves obtained after 2 a.m. ^{3}H-TdR injections and (2) by the fact that the last experimental points obtained at the 27th hr are significantly lower in groups injected at 2 a.m. In the other modalities, the values are not different between each others at this time.

DISCUSSION

The present work confirms our previous observations on diurnal fluctuations of proliferation in two methylcholanthrene induced sarcomas of C57BL male mice: the mitotic indices are at their nadir either at midnight or between midnight and 4 a.m., at time where the labelling indices reach their peak.

The purpose of the present experiments was to determine by the PLM method which diurnal change in the cell cycle could be responsible for these fluctuations of the mitotic and labelling indices.

However, the use of the PLM method to determine the transit times in a system which is neither asynchronous nor in a steady state deserves comment [38–41]. This method was

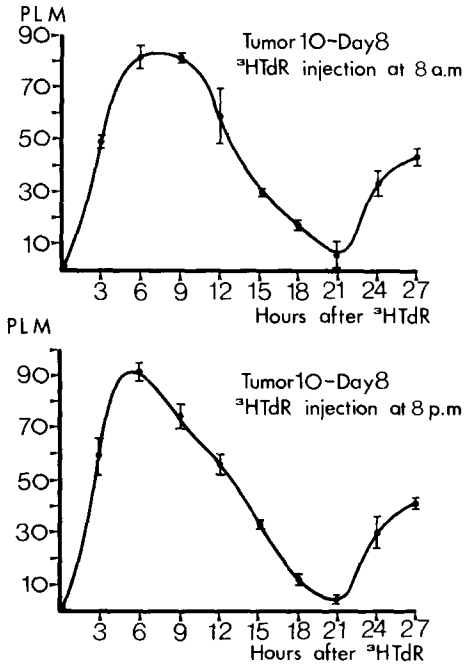

Fig. 4a.

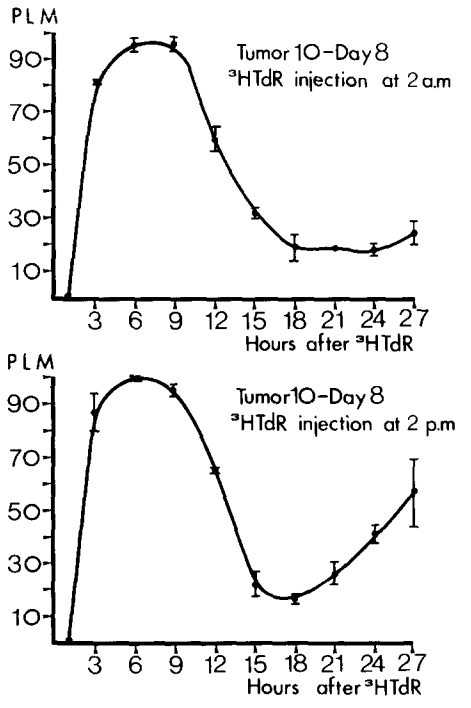

Fig. 4b.

Fig. 4. Evolution of the PLM curve in T_1 *at* day 8 *after a* ^{3}H-*TdR injection performed at* 2 a.m., 2 p.m. (*Fig. 4a*) 8 a.m. *and* 8 p.m. (*Fig. 4b*). *Each point represents the mean of* 3–5 *measurements; vertical bars the standard error of this mean.*

Table 1. *Estimated cell cycle parameters of MCA induced sarcomas at* day 8 (50 % *intercept method*)

	Hour of ^{3}H-TdR injection	$TG_1+M/2$	TS	$TG_2+M/2$	TC
T_9	2 a.m.	10.5	10.0	4.4	24.9
	8 a.m.	1.8	9.8	6.6	18.2
	2 p.m.	4.6	12.0	4.0	20.6
	8 p.m.	2.8	8.8	6.8	18.4
T_{10}	2 a.m.	19.0*	10.4	2.0	31.4
	8 a.m.	11.6	9.8	3.0	24.4
	2 p.m.	11.0	11.0	2.0	24.0
	8 p.m.	13.0	10.4	2.6	26.0

*Rough estimation by extrapolation. In practice, precisely unevaluable except by computer fitting.

first described by Quastler and Sherman [37] in the small intestine of mice, which has since then been shown to be submitted to important circadian influences [20, 28]. Except in a few selected cases [42, 43], it has been used in many tumoral systems without taking into account diurnal changes in the proliferation activity [40, 41, 44, 45] despite the fact that diurnal waves of proliferation occur in experimental and human neoplasms [26–36] as well as in normal, actively dividing tissues [1–25].

The validity of the PLM curve as a method for calculating the duration of cell cycle phases in non-asynchronous systems has been questioned especially when a single PLM curve is presented [6]. Fluctuations of the PLM evolution reflecting especially variations of the G_2 and S phases have already been described in tissues known to exhibit circadian activity such as the hamster cheek pouch [3, 11, 12, 15] or the mouse cornea [6, 18, 19] and epidermis [7, 10, 21, 22]. In the hamster cheek pouch, Möller *et al.* [15] described a variable effect of triggering cells from G_2 to M phase depending of the hour of ^{3}H-TdR injection, inducing false shortening or lengthening of G_2 phase. It was also demonstrated in such systems that the age distribution of cells in S phase [3, 6, 11, 12, 15] due to different rates of entrance or exit [3, 6, 10–12, 15, 22] present circadian fluctuations. These are also liable to influence the evolution of the PLM curve inducing false computations of G_2 and S durations.

Therefore, to obviate such induced fluctuations and obtain a better resolution of the mean duration of phase of the cell cycle, we repeated PLM curves by pulse labelling every 6th hour during a 24-hr period. For both tumours, the most significant variation was found in the duration of G_1. Successive lengthening and shortening of G_1 within a 24 hr period result in a circadian fluctuation of the flux of cells into S, realizing a partial synchrony which generates the circadian waves of L.I. and M.I. [10–12]. Although this conclusion must be confirmed by computer fitting of our results, it is in good agreement with mathematical modellings performed by Klein and Valleron [46], or Firket [47]. It is also supported by previous observations made on the hamster cheek pouch by Brown and Berry [3], Izquierdo and Gibbs [11,12] and Möller *et al.* [15], and on the mouse epidermis by Burns and Scheving [6], Grube *et al.* [10] and Tvermyr [22]. More ancient works of Bullough [4] on the 'chalone' regulation of some normal actively dividing tissues suggested also that the level of regulation lies in the period preceding DNA synthesis, and that the expression of this regulation appeared during the last hours of the rest period of animals.

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REFERENCES

1. B. Barbirolli, V. R. Potter and H. P. Morris, DNA synthesis in Morris hepatoma 9618A and in host liver following partial hepatectomy in rat adapted to controlled feeding schedules. *Cancer Res.* **32,** 7 (1972).

2. M. V. Berezkin, A comparative study of the circadian rhythms of mitotic activity in neoplasic and normal tissues (in Russian). *Bull. exp. Biol. Med.* **70,** 83 (1970).
3. J. M. Brown and R. J. Berry, The relationship between diurnal variation of the number of cells in mitosis and the number of cells synthetizing DNA in the epithelium of the hamster cheek pouch. *Cell Tiss. Kinet.* **1,** 23 (1968).
4. W. S. Bullough, Mitotic activity in the adult male mouse. *Mus musculus* L. The diurnal cycles and their relation to walking and sleeping *Proc. roy. Soc.* B **135,** 212 (1949).
5. T. J. Burns and I. F. Tannock, On the existence of a G_0 phase in the cell cycle. *Cell Tiss. Kinet.* **3,** 321 (1970).
6. E. R. Burns and L. E. Scheving, Circadian influence on the wave form of the frequency of labelled mistoses in mouse corneal epithelium. *Cell Tiss. Kinet.* **8,** 61 (1975).
7. Z. K. Cooper and J. C. Franklin, Mitotic rhythm in the epidermis of the mouse. *Anat. Rec.* **78,** 1 (1940).
8. L. B. Fischer, The diurnal mitotic rhythm in the human epidermis. *Brit. J. Derm.* **80,** 75 (1968).
9. R. F. Gasser, L. E. Scheving and J. E. Pauly, Circadian rhythm in the mitotic index of the basal epithelium and in the uptake rate of ^{3}H-thymidine by the tongue of the rat. *J. cell Physiol.* **80,** 437 (1972).
10. D. D. Grube, H. Auerbach and A. M. Brues, Diurnal variation in the labelling index of mouse epidermis. A double isotope autoradiographic demonstration of changing flow rates. *Cell Tiss. Kinet.* **3,** 363 (1970).
11. J. N. Izquierdo and S. J. Gibbs, Circadian rhythms of DNA synthesis and mitotic activity in hamster cheek pouch epithelium. *Exp. Cell Res.* **71,** 402 (1972).
12. J. N. Izquierdo and S. J. Gibbs, Turnover of cell renewing populations undergoing circadian rhythms in cell proliferation. *Cell Tiss. Kinet.* **7,** 99 (1974).
13. G. Kahn, G. P. Weinstein and P. Frost, Kinetics of human epidermal cell proliferation: diurnal variations. *J. invest. Derm.* **50,** 459 (1968).
14. S. G. Mamontov, Diurnal rhythm of mitoses in the epithelium of the mouse tongue. *Bull. exp. Biol. Med.* **66,** 1277 (1968).
15. U. Möller, J. K. Larsen and M. Faber, The influence of injected tritiated thymidine on the mitotic circadian rhythm in the epithelium of the hamster cheek pouch. *Cell Tiss. Kinet.* **7,** 231 (1974).
16. C. Pilgrim, W. Erb and W. Maurer, Diurnal fluctuations in the numbers of DNA synthesizing nuclei in various mouse tissues. *Nature* (*Lond.*) **199,** 863 (1963).
17. H. Pullman, K. J. Lennartz and G. K. Steigleder, *In vitro* examination of cell proliferation in normal and psoriatic epidermis with special regard to diurnal variations. *Arch. Derm. Forsch.* **250,** 177 (1974).
18. L. E. Scheving and J. E. Pauly, Circadian phase relationship of ^{3}H-thymidine uptake, labeled nuclei, grain counts and cell division rate in rat corneal epithelium. *J. cell Biol.* **32,** 677 (1965).
19. L. E. Scheving and J. J. Chiakulas, Twenty-four hour periodicity in the uptake of tritiated thymidine and its relation to mitotic rate in urodele larval epidermis. *Exp. Cell Res.* **39,** 161 (1965).
20. C. P. Sidgestad, J. Bauman and S. W. Lesher, Diurnal fluctuations in the number of cells in mitosis and DNA synthesis in the jejunum of the mouse. *Exp. Cell Res.* **58,** 159 (1969).
21. E. M. F. Tvermyr, Circadian rhythms in epidermal mitotic activity. Diurnal variations of the mitotic index, the mitotic rate and the mitotic duration. *Virchows Arch., Abt. B.* **2,** 318 (1969).
22. E. M. F. Tvermyr, Circadian rhythms in hairless mouse epidermal DNA synthesis as measured by double labelling with ^{3}H-thymidine (^{3}H-TdR). *Virchows Arch., Abt. B.* **11,** 43 (1972).
23. C. S. Potten, B. A. Jessup and M. B. Croxson, Incorporation of tritiated thymidine into the skin and hair follicles. II. Daily fluctuations in ^{3}H-TdR and ^{3}H-UR levels. *Cell Tiss. Kinet.* **4,** 413 (1971).
24. H. Barbason and P. Lelievre, Influence du rythme de l'activé circadienne sur les différentes phases du premier cycle cellulaire suivant une hépatectomie partielle. *C. R. Acad. Sci.* (*Paris*) **271,** 1798 (1970).

25. W. D. Heine, E. Stöcker and H. D. Heine, Tageszeitlich Rhythmen der Zellproliferation in der Kompensatorisch regenerierenden Niere nach unilateraler Nephrektomie. *Virchows Arch., Abt. B.* **9,** 75 (1971).
26. A. F. Badran and J. M. Echave Llanos, Persistence of mitotic circadian rhythm of a transplantable mammary carcinoma after 35 generations: its bearing on the success of treatment with endoxan. *J. nat. Cancer Inst.* **2,** 285 (1965).
27. J. M. Echave Llanos and R. E. Nash, Mitotic circadian rhythm in a fast growing and a slow growing hepatoma: mitotic rhythm in hepatoma. *J. nat. Cancer Inst.* **3,** 581 (1970).
28. C. Focan, H. Barbason and E. H. Betz, Mise en évidence d'un rythme nycthéméral de la division cellulaire dans des sarcomes induits par le méthylcholanthrène. *C. R. Acad. Sci. (Paris)* **276,** 2229 (1973).
29. R. E. Nash and J. M. Echave Llanos, Twenty-four hour variations in DNA synthesis of a fast growing and a slow growing hepatoma: DNA synthesis rhythm in hepatoma. *J. nat. Cancer Inst.* **5,** 1007 (1971).
30. C. Focan and G. Pierard, Variations circadiennes de la prolifération cellulaire dans des tumeurs épidermiques. *Bull. Soc. franc. Derm. Syph.* **83,** 331 (1976).
31. C. Focan, Rythme nycthéméral de la division et chimiothérapie de synchronisation-recrutement en cancérologie clinique. *Rev. med. Liège* **31,** 461 (1975).
32. C. Focan, E. P. Malaise, J. M. Richard and M. Tubiana, Circadian variations of labelling indices in oral squamous cell carcinomas. (In preparation).
33. M. Garcia Sainz and F. Halberg, Mitotic rhythms in human cancer re-evaluated by electronic computer programs. Evidence for chronopathology. *J. nat. Cancer Inst.* **37,** 279 (1966).
34. H. R. Nitze and H. R. Roseman, Die Beeinflussung des Zellteilungrsrhythmus bei menschlichen Tumoren. *Arch. Ohr.-, Nas.-, u. Kehlk.-Heilk.* **193,** 101 (1969).
35. E. Tahti, Studies of the effect of X-radiation on 24-hr variations in the mitotic activity in human malignant tumors. *Acta path. microbiol. scand. Suppl.* **117,** 1 (1956).
36. A. Voutilainen, Uber die 24 Stunden rhythmik der Mitosen frequenz in malignen Tumoren. *Acta path. microbiol. scand. Suppl.* **99,** 1 (1953).
37. H. Quastler and F. G. Sherman, Cell population kinetics in the intestinal epithelium of the mouse. *Exp. Cell Res.* **17,** 420, (1959).
38. M. Takahashi, Theoretical basis for cell cycle analysis. I. Labelled mitosis wave method. *J. theor. Biol.* **13,** 202 (1966).
39. M. Takahashi, Theoretical basis for cell cycle analysis. II. Further studies on labelled mitosis wave method. *J. theor. Biol.* **18,** 195 (1968).
40. M. Tubiana and E. Frindel, La cinétique des populations de cellules. In *La Cinétique de Prolifération Cellulaire*, I.N.S.E.R.M. Paris p. 145. (1971).
41. M. Tubiana, La cinétique de prolifération des tumeurs expérimentales. In *La Cinétique de Prolifération Cellulaire*, I.N.S.E.R.M. Paris p. 331 (1971).
42. M. H. L. Gibson and F. D. Bertalanffy, *In vitro* synchrony of solid B16 melanoma by cytosine arabinoside, an inhibitor of DNA synthesis. *J. nat. Cancer Inst.* **49,** 1007 (1972).
43. S. C. Rockwell and R. F. Kallman, Characteristics of a serially transplanted mouse mammary tumor and its tissue culture adapted derivative. *J. nat. Cancer Inst.* **49,** 735 (1972).
44. J. Denekamp, The cellular proliferation kinetics of animal tumours. *Cancer Res.* **30,** 393 (1970).
45. A. F. Hermens and G. W. Barendsen, Cellular proliferation patterns in an experimental rhabdomyosarcoma in the rat. *Europ. J. Cancer* **3,** 361 (1967).
46. B. Klein and A. J. Valleron, Mathematical modelling of cell cycle and chronobiology: preliminary results. *Biomedicine* **23,** 214 (1975).
47. H. Firket, Personal communication.