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Effects of Several Dimethylbenzacridines on Secondary Hamster Embryo Cells: Neoplastic Transformation*

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Abstract—*Several dimethylbenz[c]acridines and their isomers dimethylbenz[a]acridines were studied for their capacity to induce malignant transformation in secondary hamster embryo cells. Transformation was evaluated by the ability of transformed cells to provoke tumor formation in syngenic animals. 7,8-Dimethylbenz[c]acridine, 7,10-dimethylbenz[c]acridine, as well as a mixture of both, caused malignant transformation of hamster embryo cells, but not 7,9-dimethylbenz[c]acridine. Cultures treated with 10,12-dimethylbenz[a]acridine and 9,12-dimethylbenz[a]acridine did not become malignant and showed a decreased life-span* in vitro. *Untreated control cells retained their original characteristics and when injected in animals did not produce tumors throughout the whole experiment.*

INTRODUCTION

THE dimethylbenz[c]acridines are synthetic compounds which were primarily prepared in order to evaluate their tumorigenicity. They are of interest to cancerologists since they may be considered as analogues of the dimethylbenzanthracenes. Some of them have been shown to be highly carcinogenic. Although they are not present in the environment, unlike the hydrocarbons they resemble, they represent a type of potentially dangerous compounds.

The sarcomatogenic [1], transforming [2] and mutagenic [3] properties of 7,10-dimethylbenz[c]acridine were established using samples of the substance composed in fact, of a mixture containing 85% 7,10-dimethylbenz[c]acridine (7,10[c]) and 15% 7,8-dimethylbenz[c]acridine (7,8[c]) [4]. Univocal synthesis of these two isomers yielded small quantities of the pure substances which enabled us to study *in vitro* their respective neoplastic transforming activities on Syrian hamster embryo cells. In the same cell system, we also studied the effects of 7.9-dimethylbenz[c]acridine (7,9[c]), known to be carcinogenic *in vivo* [1], and of two other isomers, 9,12-dimethylbenz[a]acridine (9,12[a]), and 10,12-dimethylbenz[a]acridine (10,12[a]). It was shown by painting and subcutaneous injection [1] that 9,12[a], like many other methylbenz[a]acridines, is not carcinogenic in mice. The biological properties of 10,12[a] have not yet been studied.

Cell cultures offer a simplified model system for studying chemical carcinogenesis. A number of such model systems have been described in recent years [5–8]. Most of them involve the use of fibroblast cultures of either early passage diploid strains or established lines of various rodent cells. Among the cell systems used secondary Syrian hamster embryo cells have many advantages for studying neoplastic transformation *in vitro*. They are stable diploid, have a low incidence of spontaneous transformation, and can be induced to undergo neoplastic changes by chemical carcinogens [5, 6, 9, 10]. Like most fibroblast systems, the secondary Syrian hamster cells appear to have the required mixed function oxidase enzymes to metabolize the polycyclic aromatic hydrocarbons to the active forms [11, 12]. Several recent observations do imply that benzacridines are activated to carcinogenic or mutagenic products in a fashion similar to benz[a]anthracene and other aromatic hydrocarbons [13, 14].

In the present work, we studied the neoplastic transformation induced by dimethylbenz-

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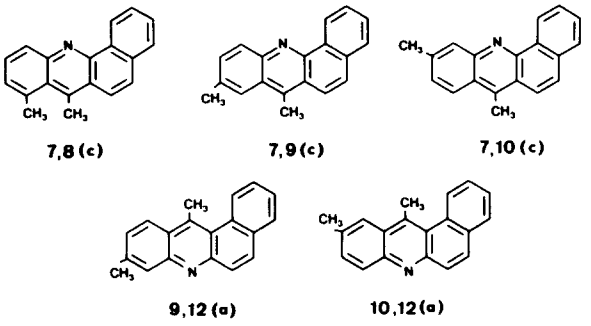
acridine treatment in secondary hamster embryo cells and the effect of such treatment on the life span of cell cultures.

MATERIALS AND METHODS

Chemicals (see Scheme)

7,8[c] and 7,10[c] were prepared according to published techniques [4]. The compounds 7,9[c] ($F=166°$; thin layer chromatography



Scheme. Chemical structure of dimethylbenzacridines used in this study.

on silica plates, elution with benzene; $R_f=0.50$), 9,12[a] ($F=146°$; thin layer chromatography on silica plates, elution with 99:1 chloroform–methanol; $R_f=0.58$) and 10,12[a] ($F=141°$; chromatography on silica gel, elution with ether; $R_f=0.87$; elution with chloroform; $R_f=0.17$) were obtained using a modified version of Benthsen's method [15] and purified by chromatography on silica columns eluted with benzene. The infra-red and nuclear magnetic resonance spectra as well as analysis of composition corresponded to the structures assigned to the compounds.

The chemicals were dissolved in acetone. The final concentration of the solvent in the culture medium did not exceed 0.5%.

Cell cultures

Primary cultures were prepared by trypsin dissociation of minced fresh Syrian hamster embryo tissue, taken between the 12th and 14th days of gestation. In all experiments, the same pool of embryos was used as the source of target cells. Hamster cells were grown in Eagle's MEM medium, supplemented with glutamine, non-essential amino acids (Grand Island Biological Co., Bio-Cult, Glasgow, Scotland), 5% fetal calf serum and 5% newborn calf serum (Flow Laboratories, U.K.), buffered with 20 mM Hepes (Calbiochem, California, U.S.A.). Antibiotics were added to the medium to the final concentrations as follows: penicillin G 100 units/ml: and streptomycin 100 μg/ml (Specia, France). Cells were cultured in 75 cm^2 Falcon plastic flasks (Falcon Plastics, Oxnard, California, U.S.A.).

Transformation assay

The medium of semiconfluent secondary cultures was replaced by a medium containing various dimethylbenzacridines at different concentrations chosen according to the results of the toxicity tests (see below). The cells were treated with the chemicals for 24 hr. Then, the cells were re-fed with a chemical-free medium, incubated and regularly observed for morphological alterations. Control cultures, fed for 24 hr with a medium containing 0.5% acetone, were handled as the chemically treated cultures.

During the first months of culture, the growth rates of both the untreated and chemically treated cells were very low and subcultures were made by splitting them at ratio 1:2. After morphological transformation, the growth rate increased and the transformed cells were subcultured at a ratio of 1:4 to 1:10, every 3–4 weeks.

The treated and untreated cells were regularly tested for their ability to produce tumors in syngenic animals. To do this, cells from 7- to 10-day-old cultures were trypsinized, counted, and 1×10^6 cells were injected (in a volume of 0.05 ml of PBS) into 2- to 3-week-old Syrian hamsters bred in the laboratory of Dr. Chouroulinkov (CNRS, Villejuif, France). The animals were examined weekly and observed for at least 5–6 months. A cell culture was considered malignantly transformed if 10^6 viable cells injected subcutaneously into animals led to a progressive, infiltrative growth. On histopathological examination all tumors proved to be fibrosarcomas.

Toxicity assays

We evaluated the toxicity of the various compounds by measuring the growth inhibition of treated cell cultures. Two-day-old secondary cells, seeded at a density of 2×10^5 cells per Falcon flask (surface 25 cm^2), were exposed to various dimethylbenzacridines at concentrations from 0.5 to 25 μg/ml for 24 hr. The medium was then replaced by fresh dimethylbenzacridine free medium, and the cells were incubated at 37°C. Previous experiments showed that ratio of number of cells in treated and untreated cultures remained stable between 5 and 14 days after the treatment. In the experiments described here the cells were

incubated, after the treatment, for 7 days; the cells were then trypsinized and counted using a Coulter electronic counter model ZBI (Coultronic, France S.A.) in treated over those in untreated cultures (Table 2). The mean number of cells was evaluated by the ratio of cell number.

Agar suspension assay

The techniques used were similar to those of Macpherson and Montagnier [16]. Trypsin-dispersed cells (1×10^5 to 2.5×10^5) were suspended in 1.5 ml growth medium containing 0.33% agar (Difco Laboratories) and seeded in a 25 cm^2 Falcon flask over a basal layer of 0.5% agar. Cells were incubated for approximately 3 weeks at 37°C and then scored for the presence of colonies.

RESULTS

To determine the concentrations of different dimethylbenzacridines usable for malignant transformation assays, we measured the cytotoxicity of the compounds in the same experimental conditions as the ones used for transformation assays. The cytotoxicity in mass cultures was measured as a reduction of growth or total population in the chemically treated groups over untreated ones. In our experimental system this method is all the more useful as the plating efficiency of secondary Syrian hamster cells is very low. The surviving fraction was determined seven days after a 24-hr exposure to 0.5–25 μg/ml of 7,8[c], 7,10[c], 7,9[c] and 10,12[a]. At equal concentrations of the compounds there were no appreciable differences in the toxicity on hamster embryo cells. In Fig. 1, we report the survival obtained after treatment with the compounds at concentrations used in the transformation experiments. It is seen that treatment at concentrations of 2, 6 and 10 μg/ml of these compounds result in surviving fractions between 0.3 and 0.9. These results were in accordance with results obtained in earlier experiments concerning the toxicity of the mixture of 7,8[c] and 7,10[c] and that of the 9,12[a] on secondary hamster embryo cells. These observations lead us to use the concentrations of 2, 6 and 10 μg/ml of all dimethylbenzacridines in the experiments described below.

The *in vitro* life span of treated and untreated cells is reported in Fig. 2. The untreated control cells, which could be maintained in culture during the whole experiment, have already reached 20 generations with a stable growth rate. Under the same

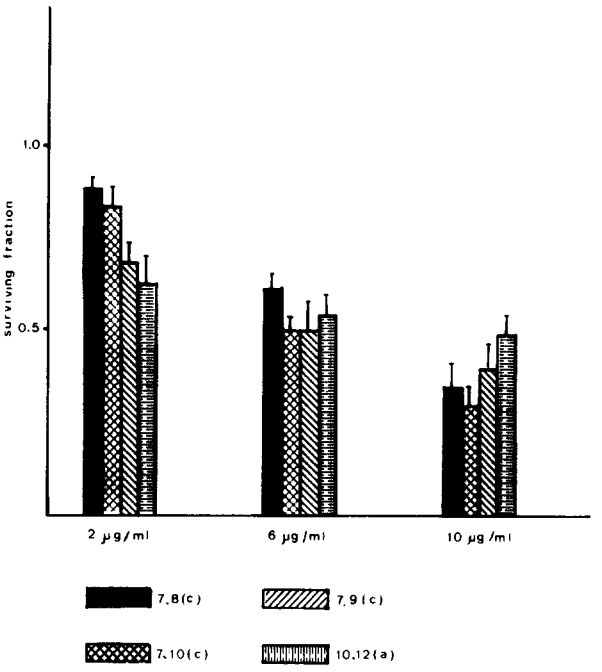


Fig. 1. Effect of dimethylbenzacridine treatment on the survival of hamster embryo cells. The survival fraction was evaluated 7 days *after treatment and calculated as*

$$\frac{\text{Number of treated cells}}{\text{Number of untreated cells}}$$

The number of cells in untreated cultures after 7 days *reached on average* 1.2×10^6 cells/flask.

experimental conditions, the growth potential of the cells treated with 2 μg/ml of 9,12[a] and 10,12[a] could not be maintained and the cells survived only 10–12 cell generations. Cell cultures treated with higher doses of these compounds declined more rapidly (after 3–6 generations). With the exception of 7,8[c]-treated cultures, we observed a rapid decrease of the growth rate in all the cell cultures treated with 10 μg/ml of all the other compounds. These latter cells did not survive more than 6–8 generations. In most of the cell cultures treated with dimethylbenz[c]acridines at concentrations of 2 and 6 μg/ml (as well as these treated with 10 μg/ml of 7,8[c]), we observed an increased rate of cell multiplication. These cells have now reached 25–30 generations.

Untreated control cells maintained under the same culture conditions as the treated ones retained their original morphology and growth pattern, and did not cause tumors in young hamsters when grafted (1×10^6 cells/animal) (Table 1). Although cultures treated with 2 μg/ml of 9,12[a] have reached 10–12 generations, their growth rate was very low, and a number of cells sufficient for grafting

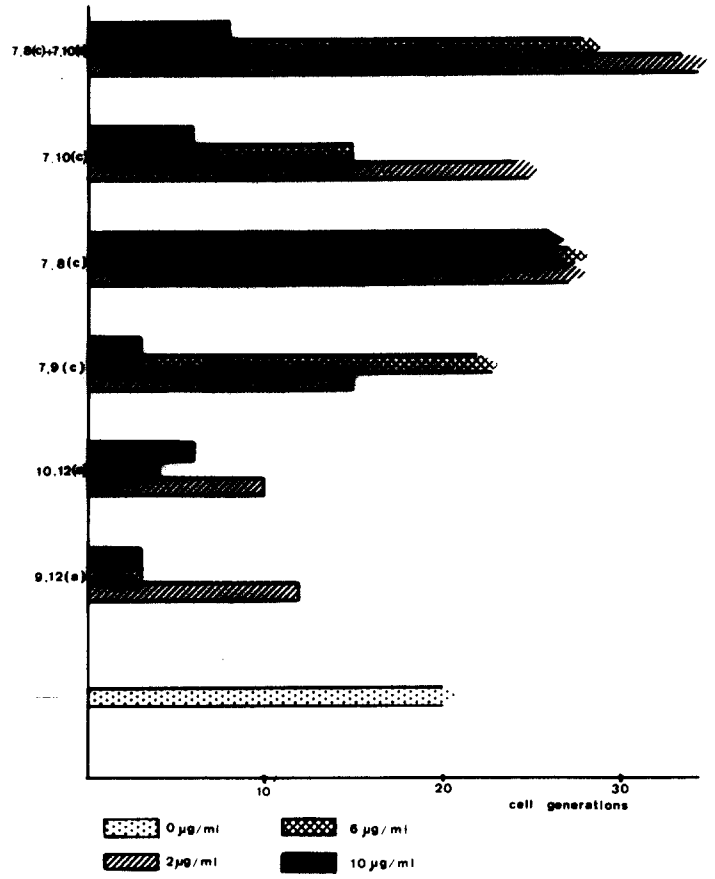


Fig. 2. Effect of dimethylbenzacridine treatment on the cellular life-span.

could be harvested only in the case of cells treated with 10,12[a]. These grafts were negative (Table 1). In previous experiments [17] cells originating from another pool of hamster embryo and exposed to 2 µg/ml of 9,12(a) survived *in vitro* for 17 generations; when grafted, they behaved as untreated cells.

In Table 2, we report the results concerning the tumor-producing capacity of cells treated with different dimethylbenz[c]acridines. The cultures treated initially with 6 µg/ml of 7,8[c] manifested malignancy from the 12th cell generation, whereas those treated with 2 µg/ml produced sarcomas in animals several generations later. Cells exposed previously to 10 µg/ml of the same compound, although exhibiting morphological changes generally associated with neoplastic transformation (increase in saturation density, lower serum requirement, growth in semi-solid medium) (Table 3) did not produce tumors when implanted in animals, even after 23 cell generations.

The cells treated with 2 µg/ml of 7,10[c] become malignant after 15 cell generations. However, the latent period for the appearance of tumors after injection of these cells was longer (72 days) that that for cells treated with 2 or 6 µg/ml of 7,8[c]. Cultures exposed previously to 6 µg/ml of 7,10[c] were accidentally lost at the 15th generation.

We have previously reported that cells treated with 2 µg/ml of the mixture (85% 7,10[c] and 15% 7,8[c]) displayed malignancy after 6–7 cell generations [2]. In this work, we report the tumor-producing ability of cells

Table 1. Transplantability of untreated and 10,12[a]-treated hamster embryo cells*

Cells	Number of cell generations	Transplantability†
Untreated	2–5	0/3
	7–10	0/8
	15	0/5
	18	0/7
Treated with 10,12[a] (2 µg/ml)	5–6	0/6
	8	0/4

*Non-carcinogenic in mice.

†Cells (1×10^6) were grafted subcutaneously in hamster. The animals were observed 150–180 days after grafting.

Table 2. Transplantability of dimethylbenz[c]acridine-treated hamster embryo cells

Cells cultures treated previously with		Number of cell generation	Tumorigenicity*	Average time of tumor appearance (days)
7,8[c]	2 μg/ml	6–12	0/8	—
		18	9/9	48
	6 μg/ml	7–8	0/5	—
		12	3/5	62
		24	5/5	21
	10 μg/ml	4–8	0/12	—
		15	0/7	—
		22–23	0/9	—
7,10[c]	2 μg/ml	4	0/6	—
		12–14	0/20	—
		15	5/5	77
		20	6/6	72
Mixture	7,8(c)+7,10(c) 6 μg/ml	5–8	0/12	—
		10	5/5	38
		26	8/8	21
7.9[c]	2 μg/ml	6	0/5	—
		9	0/6	—
		13	0/3	—
	6 μg/ml	6–9	0/5	—
		22	0/5	—

*Cells (1×10^6) were grafted subcutaneously in hamster. The animals were observed 150–180 days after grafting.
†Cells treated with 2 μg/ml produce tumors in animals after 6–7 cell generations [2].

previously treated with 6 μg/ml of the same mixture (85% 7,10[c] and 15% 7,8[c]). The acquisition of malignancy by hamster cells treated with 6 μg/ml of the mixture was observed earlier (i.e. after 10 cell generations) than when the cells were treated with each of the constituents of the mixture.

In contrast to the above reported results on the transforming capacity of the two dimethylbenz[c]acridines as well as the mixture of the two, we could not detect in our experimental system any transforming activity of 7,9[c]. Cells exposed to 2 and 6 μg/ml of this compound, derived from the same pool of hamster embryos and maintained in the same culture conditions as all the other treated cultures, have not yet displayed any sign of malignant or morphological transformation even after 22 generations for cells treated with 6 μg/ml (Table 2).

Table 3. Growth properties of untreated and 7,8(c)-treated hamster embryo cells and their tumorigenicity

Cells* treated previously with	Saturation density (cells × 10^4/cm^2)	Doubling time (hr)	Saturation density in medium containing only 2% serum (cells × 10^4/cm^2)	Growth in soft agar	Tumorigenicity
—	1.8	46	0.4×10^4	—	—
7,8[c] 2 μg/ml	6.4	42	2.5×10^4	++	+
7,8[c] 6 μg/ml	9.8	22	Not tested	+	+
7,8[c] 10 μg/ml	8.4	16	3.6×10^4	+	−
7,8[c]+7,10[c] 6 μg/ml	14.5	19	9×10^4	+	++

*17th cell generation.

DISCUSSION

Only a few studies on the transforming and mutagenic activity of benzacridines have been performed [2, 3, 18, 19], even though they form a homogeneous class of compounds, the carcinogenic properties of which have been systematically studied *in vivo*, and for which structure–activity correlations have been sought [14, 20].

In this report, we present results concerning the neoplastic transforming activity of several dimethylbenzacridines on secondary Syrian hamster embryo cells. As criteria of neoplastic transformation we used the tumor-producing capacity of cells when implanted in syngenic hosts. In such an experimental system, we observed previously [2], and this study confirms it, that the carcinogenic mixture of 85% 7,10[c] and 15% 7,8[c] has a high transforming activity. The present results demonstrate that both constituents of this mixture, the pure substances 7,8[c] and 7,10[c], induce malignant cell transformation. Cells previously treated with these compounds were able to produce tumors in animals, 12–18 cell generations after the treatment (Table 2); whereas, untreated control cells did not produce tumors in animals throughout the experiment (Table 1).

Under the same experimental conditions, hamster embryo cell exposed to 7,9[c] showed neither signs of malignant transformation nor morphological transformation. Only the fact that the 7,9[c]-treated cells could be maintained in culture for a prolonged period may indicate a possible initiation of a transforming process.

It is interesting to note that 7,9[c], like the mixture (85% 7,10[c] and 15% 7,8[c]), is tumorigenic in mice [1], and is able to transform rat cells infected with Rauscher leukemia virus [19] and two established cell lines: BHK 21 (baby hamster kidney cells) and WI 38 (human lung cells) [3]. The differences between the cell systems and/or transformation criteria used may not be sufficient to account for the discrepancy between our results on 7,9[c] transforming activity and those cited above.

The concentrations of all dimethylbenzacridines used in our transformation experiments were chosen so that the toxicity effects on secondary cells would be similar. It is possible that those of 7,9[c] were not optimal for the expression of the carcinogenic potential of this compound, and that the concentrations of 2 and 6 μg/ml are already excessive in this respect. Indeed, experiments reported by Freeman *et al.* in a rat cell system [19] showed that treatment with 1 μg/ml of 7,9[c] give more and better defined foci than with 5 μg/ml; 10 μg/ml given negative results; and 50 and 100 μg/ml were toxic.

In addition it is interesting to note the effects of dimethylbenzacridine treatment on the *in vitro* cellular life-span. It is now generally accepted that diploid mammalian cells have a limited ability to replicate *in vitro*, which may reflect senescence at the cellular level [21–24]. Starting with a primary culture of cells from a pool of Syrian hamster embryo, cells multiply at a constant rate for several generations. Afterwards, two possibilities exist for normal untreated cells: a rapid decline or a series of evolutionary changes expressing various new properties leading often to spontaneous malignant transformation. In order to have a negative control for transformation assays, the normal untreated hamster cells should maintain their original growth pattern and morphology as long as possible. The data above suggest that Syrian hamster embryo cells cultured at a low growth rate can reach 18–20 cell generations without important changes in their original patterns.

Concerning the effect of a carcinogenic treatment on the *in vitro* life-span of diploid mammalian cells, Huberman *et al.* reported that the treatment of hamster embryo cells with the carcinogen dimethylnitrosamine results in an increase in cell multiplication and in cellular life-span [25]. From the present experiments it appears that the treatment with dimethylbenz[c]acridines also increases both the capacity of cell multiplication and the cellular life-span, even in the case of doses of carcinogen for which no malignant transformation was demonstrated here (for example, 7,8[c] (10 μg/ml)-treated cells and 7,9[c] (6 μg/ml)-treated cells).

In contrast, we observed that the *in vitro* treatment with 9,12[a], the *in vivo* non-carcinogenic property of which has been already established, and also with 10,12[a] decreases the rate of cell multiplication and the cellular life span, even with doses which do not produce important cytotoxicity. The latter observations can be related to those described by Lima *et al.* [26] showing that a reduction in life-span and premature senescence can be induced in diploid cells *in vitro* by exposure of early passage cultures to low dose rate irradiation.

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REFERENCES

1. A. Lacassagne, N. P. Buu-Hoi, R. Daudel and F. Zajdela, The relation between carcinogenic activity and the physical and chemical properties of angular benzacridines (and references cited within). *Advanc. Cancer Res.* **4,** 315 (1956).
2. P. Markovits, J. Coppey, D. Papadopoulo, A. Mazabraud and M. Hubert-Habart, Transformation maligne de cellules d'embryon de hamster en culture par la dimethy-7,10 benzo[c]acridine. *Int. J. Cancer* **14,** 215 (1974).
3. I. F. H. Purchase, E. Longstaff, J. Ashby, J. A. Styles, D. Anderson, P. A. Lefevre and F. R. Westwood, An evaluation of 6 short-term tests for detecting organic chemical carcinogens. *Brit J. Cancer* **37,** 873 (1978).
4. C. Pene, J. M. Lhoste, P. Markovits and M. Hubert-Habart, Synthesis of 7,10- and 7,8-dimethylbenz[c]acridine. *J. chem. Soc., Perkin* **1,** 2277 (1976).
5. Y. Berwald and L. Sachs, *In vitro* transformation of normal cells to tumor cells by carcinogenic hydrocarbons. *J. nat. Cancer Inst.* **35,** 641 (1965).
6. J. A. di Paolo and P. J. Donovan, Properties of Syrian hamster cells transformed in the presence of carcinogenic hydrocarbons. *Exp. Cell Res.* **48,** 361 (1967).
7. T. T. Chen and C. Heidelberger, Quantitative studies on the malignant transformation of mouse prostate cells by carcinogenic hydrocarbons *in vitro*. *Int. J. Cancer* **4,** 166 (1969).
8. R. Montesano, L. Saint Vincent and L. Tomatis, Malignant transformation *in vitro* of rat liver cells by dimethylnitrosamine and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Brit. J. Cancer* **28,** 215 (1973).
9. P. Markovits, P. Daudel, D. Papadopoulo, A. Mazabraud et M. Hubert-Habart, Sur la cancérisation chimique de cellules d'embryon de hamster en culture de masse. *Bull. Cancer* **62,** 59 (1975).
10. J. C. Barrett and P. O. P. Ts'o, Evidence for the progressive nature of neoplastic transformation *in vitro*. *Proc. nat. Acad. Sci. (Wash.)* **75,** 3761 (1978).
11. R. Pienta, A hamster embryo cell model system for identifying carcinogens. In *Carcinogens: Identification and Mechanisms of Action.* (Edited by A. C. Griffin and C. R. Shaw), p. 121. Raven Press, New York (1979).
12. V. Poirier, D. Papadopoulo and P. Markovits, Aryl hydrocarbons hydroxylase activity of cultured mouse and hamster cells, originating from different organs. Unpublished data.
13. I. A. Smith and P. G. Seybold, Substituent effects in chemical carcinogenesis: methyl derivatives of benzacridines. *J. heterocycl. Chem.* **16,** 421 (1979).
14. T. Okano, T. Horie, T. Koike and N. Motahashi, Relationship of carcinogenicity, and K-region reactivity in benz[c]acridines. *Gann* **70,** 749 (1979).
15. N. P. Buu-Hoi et J. Lecocq, De la chimie des inhibiteurs de carbures cancérigènes: Synthèse de dimethylbenzacridines. *C.R. Acad. Sci. (Paris)* **218,** 792 (1944).
16. I. Macpherson and L. Montagnier, Agar suspension culture for the selective assay of cells transformed by polyoma virus. *Virology* **23,** 4435 (1964).
17. D. Papadopoulo, S. Levy, L. Chamaillard, O. Beesau, M. Hubert-Habart and P. Markovits, Hamster cells, untreated and treated with chemical carcinogens, maintained *in vitro* for $2\frac{1}{2}$ years. *Brit. J. Cancer* **36,** 65 (1977).
18. H. R. Glatt, H. Schwind, F. Zajdela, A. Croisy, P. C. Jacquignon and F. Oesch, Mutagenicity of 43 structurally related heterocyclic compounds and its relationship to their carcinogenicity. *Mut. Res.* **66,** 307 (1979).
19. A. E. Freeman, E. K. Weisburger, J. H. Weisburger, R. G. Wolford, J. M. Maryak and R. J. Huebner, Transformation of cell cultures as an indication of the carcinogenic potential of chemicals. *J. nat. Cancer Inst.* **51,** 799 (1973).
20. D. B. Clayson, Carcinogenic and anticarcinogenic properties of acridines and references quoted. In *Acridine*, 2nd Edn. (Edited by R. M. Acheson), p. 815. Wiley–Interscience, New York.
21. L. Hayflick and P. S. Moorhead, The serial cultivation of human diploid cell strains. *Exp. Cell. Res.* **25,** 585 (1961).
22. L. Hayflick, The limited *in vitro* lifetime of human diploid cell strains. *Exp. Cell Res.* **37,** 614 (1965).

23. G. J. Todaro, K. Nilausen and H. Green, Growth properties of polyoma virus-induced hamster tumor cells. *Cancer Res.* **23,** 825 (1963).
24. G. J. Todaro and H. Green, Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell. Biol.* **17,** 299 (1963).
25. E. Huberman, S. Salzberg and L. Sachs, The *in vitro* induction of an increase in cell multiplication and cellular life span by the water-soluble carcinogen dimethylnitrosamine. *Proc. nat. Acad. Sci.* **59,** 77 (1968).
26. L. Lima, E. Malaise and A. Maciera-Coelho, Aging *in vitro*. Effect of low dose-rate irradiation on the division potential of chick embryonic fibroblasts. *Exp. Cell Res.* **73,** 345 (1972).