Proc. Natl. Acad. Sci. USA
Vol. 75, No. 7, pp. 3363–3366, July 1978
Genetics

Induction of ouabain-resistant mutations in C3H 10T1/2 mouse cells by ultraviolet light

(*in vitro* transformation/transformation target size)

GERALD L. CHAN AND JOHN B. LITTLE

Laboratory of Radiobiology, Department of Physiology, Harvard University School of Public Health, Boston, Massachusetts 02115

Communicated by Shields Warren, April 24, 1978

ABSTRACT **Ouabain-resistant mutants were induced in C3H mouse embryo 10T1/2 fibroblasts by exposure to ultraviolet light, thus making available an *in vitro* system for studying mutagenesis and oncogenic transformation in parallel. ^{86}Rb uptake studies showed that biochemical mutants at the plasma membrane Na^+,K^+ transport ATPase (EC 3.6.1.3) locus were being selected for in this system. The optimal expression time for the mutants was found to depend on the dose of ultraviolet light, as was the induced mutation frequency. The ratio of transformation to mutation frequencies was found to be on the order of 10 for four different doses, suggesting that the target size in the cellular genome for transformation may be approximately 10 times the size of the Na^+,K^+ ATPase gene. We propose that both transformation and mutation induction can now be quantitatively studied in this single system.**

There is at present a significant body of information based on both experimental investigations and theoretical considerations that supports the view that the oncogenicity of chemical and physical carcinogens is derived from their mutagenic action on the genome of animal cells (1–8). In order to elucidate the relationship between mutation induction and oncogenesis, it seems necessary, if not ultimately imperative, that experimental systems be available that are amenable to studying both processes in parallel. Animal systems are useful in such studies but, because of the time and cost involved as well as difficulties in the interpretation of the data due to uncontrollable biological variation, increasing efforts have been directed to the search for *in vitro* systems that can fulfill this goal.

The lack of such suitable systems can be ascribed in part to the independent tracks along which *in vitro* studies of mutagenesis and oncogenic transformation have developed. In the area of mutation research, sufficient evidence has now accrued to substantiate the assertion that heritable variations in cultured somatic cells induced by mutagens are caused by formal genetic mechanisms and are therefore indeed mutants (9–11). However, for obvious reasons, most studies in this field have been restricted to cell lines that have a quasidiploid chromosome complement and are functionally hemizygous at many genetic loci (10, 12). These cell lines, as examplified by the CHO and V79 lines, have been shown to have undergone extensive chromosomal rearrangement in terms of both banding pattern and sequence of replication (12, 13). Having acquired immortality and malignant potential, these cells are basically unsuitable for *in vitro* oncogenic transformation assays because they are in essence already transformed.

At the same time, although certain *in vitro* transformation systems have been developed, success has not been achieved in isolating mutants at known genetic loci. Some studies have been forced to take the unfavorable approach of comparing and correlating transformation in a mammalian cell system with mutation induction in a bacterial system (14, 15) or the former in one mammalian system and the latter in another (4, 16, 17).

More recently, with the development of the ouabain selection system for mutations at the Na^+,K^+ ATPase locus (18, 19), attempts have been made to study both mutagenesis and transformation in primary cultures of hamster embryo fibroblasts (20, 21). This system has the attractive feature of being diploid. However, being primary cultures, one has the problem of cellular senescence as well as both variability from embryo to embryo and heterogeneity of cell types from a single embryo. To avoid some of these problems, some investigators have used cryopreserved cells taken from embryos, pooled, and put into culture some time ago (15, 22). Fluctuations of this sort obviously pose limitations on the reliability of this system and on the reproducibility of results among different laboratories.

The other widely used *in vitro* transformation system has been the C3H 10T1/2 clone 8 mouse embryo fibroblast line. As a transformation system, it has been extensively characterized (23–26). These cells are extremely sensitive to postconfluence inhibition of growth. When early passage cells were used, the frequency of background transformation was below the level of detection. The experimental protocol allows transformants to be scored as foci of cells piled up on top of a monolayer of normal cells. Their altered morphology makes the scoring of transformants unambiguous. When cells from the transformed foci were injected back into syngeneic animals, there was a 100% correlation with tumorigenicity (24, 25).

Several laboratories have attempted to obtain biochemical mutants in this system and have not been successful. In this paper, we report that ouabain-resistant mutants can be induced and recovered in these cells after exposure to UV light. We present here the characterization of this mutation system and suggest that both mutagenesis and oncogenic transformation can now be reliably studied in quantitative terms in this single system.

MATERIALS AND METHODS

Cell Cultures. C3H 10T1/2 cl.8 cells were originally obtained from C. Heidelberger. They were grown in Eagle's basal medium supplemented with 10% fetal calf serum (Microbiological Associates) that had been heat-inactivated at 56° for 30 min. The selective medium used in the mutagenesis assay differed from the nonselective medium only in that it contained ouabain (Sigma) dissolved directly in the medium.

Cells were routinely passaged in Blake bottles and were maintained in 5% CO_2 incubators held at 37°. A 0.15% trypsin solution was used to remove cells from their substrate.

Mutagenesis Assay. Cells (5×10^5) obtained from stock cultures were plated in 100-mm Falcon plastic dishes in nonselective medium. After 16 hr of incubation to allow the cells

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

to attach, the medium on the plates was removed by suction and the cells were exposed without the plastic lid to a bank of five GE G8T5 germicidal lamps emitting predominantly at a wavelength of 254 nm. The exposure rate at the surface of the cells was 3.85 ergs mm^{-2} sec^{-1}. After the exposure, the plates were replenished with fresh nonselective medium and incubated at 37° for the expression of mutants. When the plates became confluent before the end of the expression period, the cells were trypsinized and replated at a low cell density to continue growth in normal medium.

At the end of the expression period, cells were trypsinized and replated in ouabain-containing medium at a density of 2.5 × 10^5 cells per 100-mm plate. This cell density has been found to be optimal for the recovery of mutants. Fourteen days of incubation in the same medium was allowed for the mutant cells to grow to macroscopically visible colonies. An appropriate dilution of the same cell suspension was plated in parallel in nonselective medium for the assay of clonogenic survival of these cells. At the end of the 2-week period, all plates were fixed with Bouin's solution and stained with trypan blue. Mutation frequency was computed on a per survivor basis.

Rubidium Uptake Assay. Approximately 10^5 cells were seeded in glass scintillation vials in 2 ml of nonselective medium. After 24 hr, the medium was replaced with one that contained ouabain at a specified concentration. The vials were then incubated for 3 hr to allow ouabain binding to proceed to a saturation level. A small amount of rubidium-86 (New England Nuclear) dissolved in complete medium was added to each vial to yield a final concentration of approximately 1 μCi/ml. The vials were then returned to the incubator for 15 min to allow transport of ^{86}Rb to proceed. At the end of the 15 min, the medium was removed and the vials were washed three times with ice-cold Earle's balanced salt solution. The cells were then lysed in 10 ml of distilled water, and the Cerenkov radiation from the radioisotope retained by the cells was counted in this aqueous medium with a scintillation counter set for a ^3H window.

RESULTS

Effect of Ouabain on Cell Survival. In order to determine the minimum ouabain concentration that is lethal to wild-type cells, including any unusually resistant ones that may arise by chance (27), a plating efficiency assay was performed for various concentrations of ouabain. In the range 1–100 μM, ouabain was essentially nontoxic to wild-type cells (Fig. 1). Between 0.1 and 1.0 mM, the plating efficiency decreased by 6 orders of magnitude, leveling off thereafter at higher drug concentrations. Based on these data, a ouabain concentration of 3 mM was chosen for the mutagenesis assay. At this drug concentration, only the spontaneously arising true mutants, which existed at a frequency of 2.5 × 10^{-7} in a random population of wild-type cells, survived to form macroscopic colonies. Such colonies were found on plates with a background that was virtually devoid of any attached cells. The colonies appeared healthy on the basis of cell morphology, and the colony sizes at the end of the 2-week selection period were comparable to those of wild-type cells grown in nonselective medium.

Mutagenesis by UV Light. UV light-irradiated cells were grown in nonselective medium for various expression times before being exposed to 3 mM ouabain for mutant selection. The optimal expression time that allowed the maximal recovery of mutants was found to depend on the dose of UV light (Fig. 2), increasing from 2 days for 25 and 50 ergs/mm^2 to 6.5 days for 100 ergs/mm^2. The rate of appearance of mutants at less-than-optimal expression times was in each case greater than the rate of disappearance of mutants at longer-than-optimal expression times.

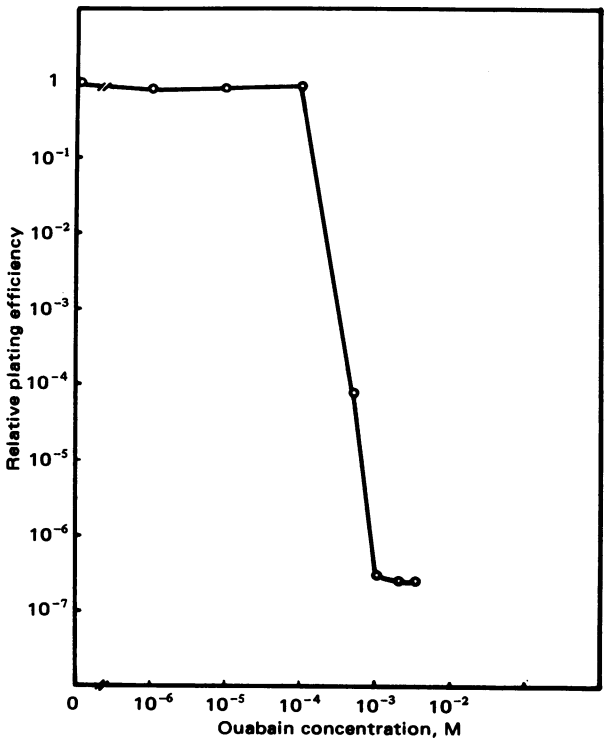

FIG. 1. Toxicity of ouabain to random populations of wild-type 10T1/2 cells. The absolute plating efficiency in ouabain-free medium was 13%.

When the mutation frequency at each optimal expression time was plotted against UV light dose, the data points followed a straight line on a semilogarithmic plot (Fig. 3). The least squares regression line that fits these data is described by the equation $\log f = 0.013D - 5.257$ in which f is the mutation

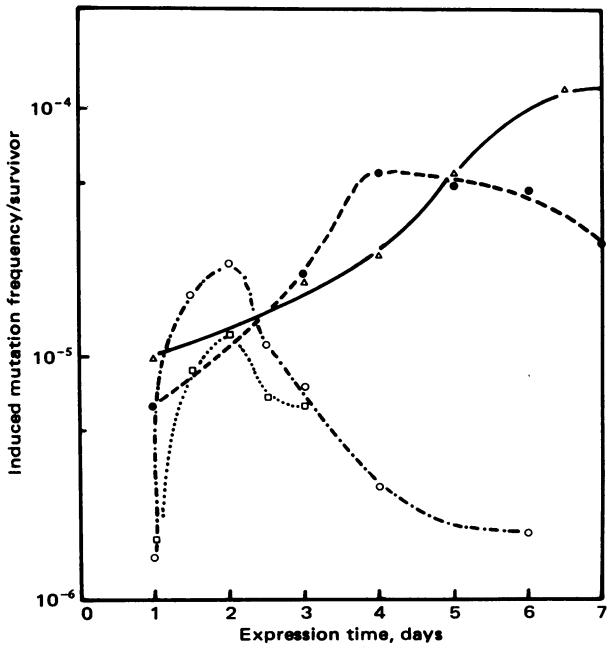

FIG. 2. Frequency of induced mutation to ouabain resistance as a function of expression time for UV light doses of 25 (□), 50 (○), 75 (●), and 100 (△) ergs/mm^2.

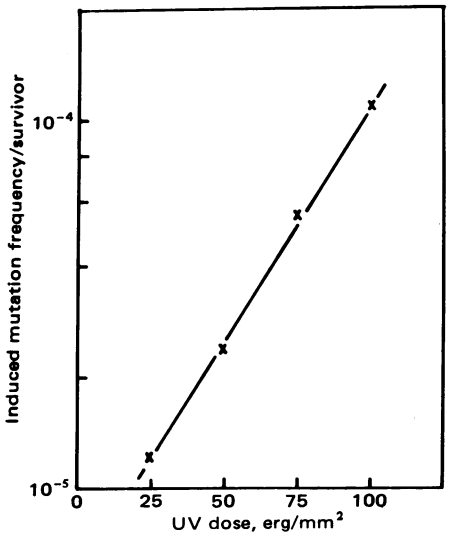

FIG. 3. Dose-response relationship for the induction of ouabain resistant mutants in 10T1/2 cells by UV light.

frequency per surviving cell and D is the dose in ergs/mm^2. The correlation coefficient is 0.99.

Ouabain Binding and Rubidium Uptake. Ouabain has been shown to inhibit specifically the function of plasma membrane Na^+,K^+ transport ATPase (ATP phosphohydrolase, EC 3.6.1.3) (28). We used the uptake of ^{86}Rb, a radioactive conjoiner of potassium, to assay the degree of inhibition of this enzyme by ouabain binding in both wild-type and mutant cells. The three UV-induced mutant cell lines used here (designated OR12, OR15, and OR16) were independently isolated by the steel cylinder method from separate plates. Mutant clones thus isolated were subsequently recloned once and passaged routinely in selective medium.

Fig. 4 illustrates the effect of ouabain concentration on ^{86}Rb uptake. At 10 mM ouabain, ^{86}Rb transport in wild-type cells was decreased to one-fifth that of cells grown in ouabain-free medium, with 50% inhibition occuring at about 1 mM. In contrast, all three mutant cell lines tested showed less than 20% inhibition of uptake for all ouabain concentrations used. These data indicate that the ouabain-resistant clones that we have isolated were indeed cells with their Na^+,K^+ ATPase enzyme altered in such a way that its affinity to ouabain was substantially decreased without significantly impairing its transport function.

DISCUSSION

We have demonstrated in these experiments that ouabain-resistant mutants can be induced by UV light in C3H 10T1/2 mouse embryo fibroblasts. The ^{86}Rb uptake studies (Fig. 4) provide firm evidence that the resistant clones we have isolated were indeed mutants with an altered gene product and not merely phenotypic variants. Moreover, the alteration in this gene product, the plasma membrane Na^+,K^+ ATPase, was a specific type that decreased its affinity to ouabain binding but maintained its ability to perform ion transport functions. In the absence of studies with the isolated enzyme such as have been done for mutants at the hypoxanthine phosphoribosyltransferase locus (29–32), the ^{86}Rb uptake assay currently provides the most conclusive demonstration of mutation at the Na^+,K^+ ATPase locus.

It should be pointed out that the quantitative nature of this mutation system is subject to certain underlying assumptions which are implicit in all mutation systems of this basic design.

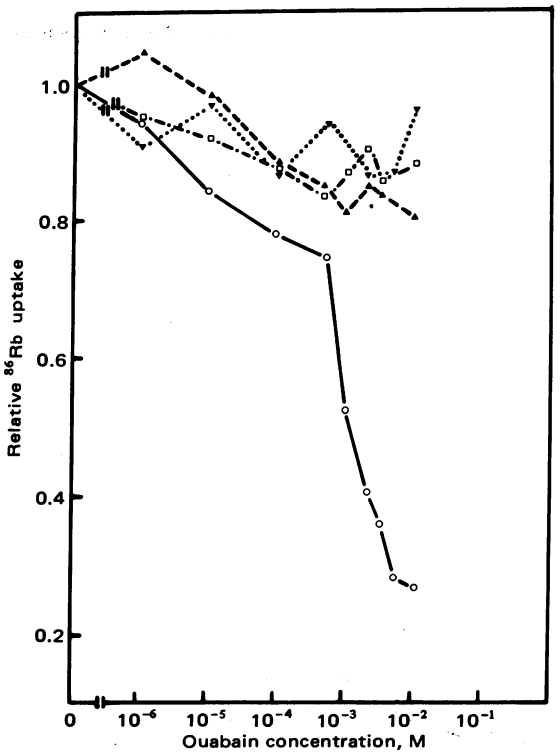

FIG. 4. Transport of ^{86}Rb in the presence of different concentrations of ouabain for wild-type and mutant 10T1/2 cells. O, Wild-type; □, mutant OR12; ▲, OR15; ▼, OR16.

First, with respect to growth rate, it has been assumed that mutants, including both those that are fully expressed and those that are not, grow at the same rate as wild-type cells during the expression period. Second, with respect to plating efficiency, it has been assumed that mutants, fully expressed or not, plate equally well as normal cells. This applies to the situations in which subculturing is required during the expression period as well as to the final plating into the selective medium. Furthermore, the estimation of the induced mutation frequencies is based on the assumption that the plating efficiency of the mutation plates (with 2.5×10^5 cells plated) is approximately the same as the plating efficiency of the survival assay plates with a few hundred to a few thousand cells plated.

The background mutation frequency of the 10T1/2 system is extremely low, on the order of 10^{-7}, and is comparable to that of other rodent cell systems reported (33, 34). This low frequency of spontaneously arising mutants renders the system sensitive even at low doses of UV light. For example, at a dose of 25 ergs/mm^2, which kills 4% of cells (26), the background mutation frequency is only 2% of the induced mutation frequency.

Our experiments here offer no explanation for the UV light dose dependence of optimal expression time beyond what has been invoked by others (34). The longer expression times required for larger doses are presumably due to more severe metabolic inhibition. Higher doses of UV light prolong the time required to complete the first DNA synthetic period after irradiation and thus induce a dose-dependent division delay (35). The disappearance of mutants for longer-than-optimal expression times is presumably due to the growth disadvantage of mutant cells. Whatever the precise mechanism may turn out to be, these data point to the critical necessity of measuring the optimal expression time for each treatment regimen when this mutation system is used.

Because mutation to ouabain resistance is a codominant

Table 1. Comparison of transformation and mutation frequencies induced by UV light in 10T1/2 cells

UV dose, ergs/mm^2	Transformation frequency (t) $\times 10^4$	Mutation frequency (m) $\times 10^5$	t/m
25	1.2	1.2	10
50	3.1	2.5	12
75	8.7	5.2	17
100	9.6	11	9

The values of t are taken from ref. 25. The values for m are taken from Fig. 3.

marker (18) likely to be of the point mutation type (34), one can compare the mutation frequencies obtained in these experiments to the transformation frequencies obtained in the same system (26) to arrive at an estimation of the target size for oncogenic transformation. Table 1 presents the results of such comparisons for four different UV light doses. In each case, the ratio of transformation to mutation frequencies is on the order of 10. These values are similar to those obtained independently by two different laboratories by treating primary hamster embryo cells with reactive derivatives of chemical carcinogens (20, 21).

It is tempting to infer from these results that the target size for transformation in the 10T1/2 cells is 10 times that of the Na^+,K^+ ATPase gene, assuming that interaction of UV light with all parts of the cellular genome is random. This straightforward interpretation, however, is likely to lead to an overestimation of the transformation target size because recent data have shown that mutations to ouabain resistance involves only a restricted region of the 97,000 dalton subunit of the Na^+,K^+ ATPase enzyme (36, 37). In other words, the size of the mutation target is not that of the whole gene for the Na^+,K^+ ATPase enzyme, which has a molecular weight of 250,000 (38), but is a small fraction of it. Until we know the size of the region of the polypeptide that is responsible for altered ouabain binding, we cannot arrive at an accurate determination of the size of the transformation target. As a first approximation, these considerations suggest that the transformation target could be as small as one to a few average-size genes.

We propose that the mouse embryo 10T1/2 fibroblast system can be used to study quantitatively both mutagenesis and oncogenic transformation by various chemical and physical carcinogens. Although ouabain-resistant mutants have not been induced by x-ray in any cell system, chemical carcinogens such as ethylmethane sulfonate, which has been shown to be x-ray mimetic in terms of DNA damage and repair (39), have been shown to induce ouabain-resistant mutants in Chinese hamster cells (34). It can be expected, therefore, that this system should be useful for a wide spectrum of carcinogens. Furthermore, mutation to ouabain resistance may be a good index for mutations at other genetic loci because Arlett *et al.* (34) have demonstrated a broad agreement between results obtained from the ouabain and 8-azaguanine selection systems. Quantitative study of mutagenesis and transformation in the same experimental system may yield more interpretable data that would be helpful in the elucidation of the mechanisms of carcinogenesis.

We are most grateful to Dr. R. M. Baker for many helpful discussions. This study was supported by Research Grants CA-11751 and ES-00002 and Training Grant CA-09078 from the National Institutes of Health.

1. McCann, J. & Ames, B. N. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 950–954.
2. Ames, B. N. (1976) *Science* **191,** 241–245.
3. Miller, E. C. & Miller, J. A. (1971) in *Chemical Mutagens: Principles and Methods for their Detection,* ed. Hollaender, A. (Plenum, New York), Vol. 1, pp. 83–119.
4. Huberman, E. & Sachs, L. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 188–192.
5. Burnet, F. M. (1974) in *Chromosomes and Cancer,* ed. German, J. (Wiley, New York), pp. 21–38.
6. Knudson, A. G. (1973) *Adv. Cancer Res.* **17,** 317–352.
7. Chu, E. H. Y., Trosko, J. E. & Chang, C. (1977) *J. Toxicol. Environ. Health* **2,** 1317–1334.
8. Cairns, J. (1975) *Nature* **255,** 197–201.
9. DeMars, R. (1974) *Mutation Res.* **24,** 335–364.
10. Siminovitch, L. (1976) *Cell* **7,** 1–11.
11. Littlefield, J. W. (1976) *Variation, Senescence, and Neoplasia in Cultured Somatic Cells* (Harvard University Press, Cambridge, MA).
12. Deaven, L. & Peterson, D. (1973) *Chromosoma* **41,** 129–144.
13. Worton, R. G., Ho, C. C. & Duff, C. (1977) *Somat. Cell Genet.* **3,** 27–45.
14. Kondo, S. (1977) *Br. J. Cancer* **35,** 595–601.
15. Pienta, R. J., Shah, M. J., Lebherz, W. B. & Andrews, A. W. (1977) *Cancer Lett.* **3,** 45–52.
16. Huberman, E. & Donovan, P. J. & DiPaolo, J. A. (1972) *J. Natl. Cancer Inst.* **48,** 837–840.
17. Huberman, E. & Sachs, L. (1974) *Int. J. Cancer* **13,** 326–333.
18. Baker, R. M., Brunette, D. M., Mankovitz, R., Thompson, L. H., Whitmore, G. F., Siminovitch, L. & Till, J. E. (1974) *Cell* **1,** 9–21.
19. Mankovitz, R., Buchwald, M. & Baker, R. M. (1974) *Cell* **3,** 221–226.
20. Huberman, E., Mager, R. & Sachs, L. (1976) *Nature* **264,** 360–361.
21. Barrett, J. C., Crawford, B. D. & Ts'o, P. O. P. (1976) *J. Cell Biol.* **70,** suppl., 233a.
22. Pienta, R. J., Poiley, J. A. & Lebherz, W. B. (1977) *Int. J. Cancer* **19,** 642–655.
23. Reznikoff, C. A., Bertram, J. S., Brankow, D. W. & Heidelberger, C. (1973) *Cancer Res.* **33,** 3239–3249.
24. Heidelberger, C. (1975) *Annu. Rev. Biochem.* **44,** 79–121.
25. Terzaghi, M. & Little, J. B. (1975) *Nature* **253,** 548–549.
26. Chan, G. L. & Little, J. B. (1976) *Nature* **264,** 442–444.
27. Thompson, L. H. & Baker, R. M. (1973) in *Methods in Cell Biology,* ed. Prescott, D. M. (Academic, New York), Vol. 6, pp. 209–281.
28. Schwartz, A., Lindenmayer, G. E. & Allen, J. C. (1972) in *Current Topics in Membranes and Transport,* ed. Bronner, F. & Kleinzeller, A. (Academic, New York) Vol. 3, pp. 1–82.
29. Beudet, A. L., Roufa, D. J. & Caskey, C. T. (1973) *Proc. Natl. Acad. Sci. USA* **73,** 320–324.
30. Fenwick, R. G., Jr. & Caskey, C. T. (1975) *Cell* **5,** 115–122.
31. Fenwick, R. G., Jr., Sawyer, T. H., Kruh, G. D., Astrin, K. H. & Caskey, C. T. (1977) *Cell* **12,** 383–391.
32. Milman, G., Krauss, S. W. & Olsen, A. S. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 926–930.
33. Chang, C., Trosko, J. E. & Akera, T. (1978) *Mutation Res.,* in press.
34. Arlett, C. F., Turnbull, D., Harcourt, S. A., Lehmann, A. R. & Colella, C. M. (1975) *Mutation Res.* **33,** 261–278.
35. Rauth, A. M. (1970) *Curr. Top. Radiat. Res.* **6,** 197–248.
36. Robbins, A. R. & Baker, R. M. (1977) *Biochemistry* **16,** 5163–5168.
37. Ruoho, A. & Kyte, J. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 2352–2356.
38. Dahl, J. L. & Hokin, L. E. (1974) *Annu. Rev. Biochem.* **43,** 327–356.
39. Regan, J. D. & Setlow, R. B. (1974) *Cancer Res.* **34,** 3318–3325.