Biochemical Pharmacology, Vol. 30, No. 22, pp. 3039–3044, 1981.
Printed in Great Britain.

0006-2952/81/223039–06 $02.00/0

MUTANTS OF HeLa CELLS RESISTANT TO OUABAIN AND CASSAINE: GENETIC EVIDENCE FOR THE COMMON SITE OF ACTION OF CARDIAC GLYCOSIDES AND ERYTHROPHLEUM ALKALOIDS

RADHEY S. GUPTA
Department of Biochemistry, McMaster University, Hamilton, Ontario L8N 3Z5, Canada

(*Received* 9 *February* 1981; *accepted* 13 *April* 1981)

Abstract—Stable mutants highly resistant to the cardiac glycoside ouabain (Oua^R) and the erythrophleum alkaloid cassaine (Cas^R) are obtained at similar frequencies in different experiments in mutagen-treated cultures of HeLa cells. All of the Oua^R and Cas^R mutants examined (twelve of each kind from two independent experiments) exhibited identical cross resistance patterns to ouabain and cassaine and to a number of other cardiac glycosides and aglycones such as oleandrin, strophanthidin, digitoxigenin and digitoxin, which were examined. The apparent identity of Oua^R and Cas^R mutants as suggested by these studies indicates that resistance to these two groups of compounds results from the same primary lesion, which from earlier studies on the Oua^R mutants is known to involve the plasma membrane Na^+, K^+-ATPase. Results of genetic studies support the notion that these two groups of compounds, i.e. cardiac glycosides and erythrophleum alkaloids, possess common structural determinants which are responsible for their biological activities, and mutants are selected in both cases against these common determinants. The Oua^R and Cas^R mutants, however, did not show any cross resistance to other types of inhibitors of Na^+,K^+-ATPase function such as ethacrynic acid, sanguinarine, quindonium bromide, suramin and oligomycin, indicating that the site of action of these inhibitors is different from that of cardiac glycosides and erythrophleum alkaloids.

The various cardiac glycosides, e.g. ouabain, strophanthidin and digitalis, are specific inhibitors of the plasma membrane Na^+- and K^+-stimulated adenosine triphosphatase (Na^+,K^+-ATPase) which is responsible for the active transport of these ions across the cell membrane [1–4]. Because of their cardiotonic effects in animals and humans, these compounds have been investigated extensively during the past two decades [5, 6]. The structural features which are currently considered to be essential for their biological activities are a cyclopentaphenanthrene nucleus with A/B *cis*, B/C *trans* and C/D *cis*, fusion of the four rings, a C-14 hydroxyl group, and an unsaturated lactone ring in the β-configuration on C-17 [1, 5–7]. However, the erythrophleum alkaloids (cassaine, coumingine, etc.), which do not possess any of the above structural features (see Fig. 1), also exhibit biochemical and pharmacological effects similar to those shown by cardiac glycosides [1, 6–9].

An approach that has proved very useful in establishing whether any two given compounds act at the same site is the genetic approach in which cellular mutants, resistant to the drugs, are obtained and their cross resistance patterns are examined [10–14]. If the two compounds act at the same site and owe their biological activities to the same common structural determinants, then mutants resistant to either of these compounds should behave identically. Using this approach we have recently shown that several structurally unrelated compounds belonging to different families of alkaloids, e.g. emetine and tubulosine (benzoisoquinolines), cryptopleurine (phenanthroquinolizidine) and tylocrebrine (phenanthroindolizidine), possessed common structural determinants which were responsible for their

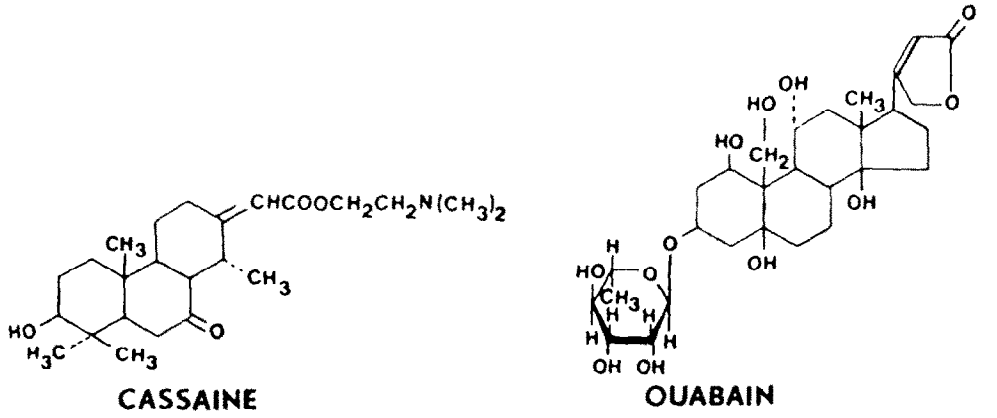

Fig. 1. Chemical structures of ouabain and cassaine.

biological activities as inhibitors of protein synthesis [10, 13]. Since both ouabain and cassaine are toxic to mammalian cells [15, 16], the above approach could be employed to establish whether these drugs act at the same site.

In the present paper, mutants of HeLa cells which have been independently selected for resistance to ouabain and cassaine are described. The cross resistance of these mutants to cassaine, ouabain, a number of other cardiac glycosides and aglycones, and several other drugs, e.g. ethacrynic acid, sanguinarine, suramin, oligomycin and quindonium bromide [see Ref. 2], which also seem to inhibit Na^+,K^+-ATPase activity, has been examined. The results show that cellular mutants selected for resistance to cassaine and ouabain are inditinguishable from each other, and provide strong evidence for a common site of action of these agents.

MATERIALS AND METHODS

Cell lines and culture conditions. The majority of the experiments in these studies were carried out with the HeLa cell line, which is a fibroblastic line that has been established from human cervical carcinoma [17]. The cells were routinely cultivated as monolayer cultures in MEM alpha medium (Grand Island Biological Co., Grand Island, NY) supplemented with 7.5% fetal calf serum and 5% newborn calf serum by procedures described earlier [18, 19]. The cell count measurements were made using a Coulter Electronic Counter (model Z_F).

Effect of drugs on cell survival (plating efficiencies). Plating efficiencies (i.e. number of colonies observed per cell plated) of cells were determined by plating a known number of cells (based on Coulter cell-counter measurement) of a single cell suspension of the culture in tissue culture dishes containing the growth medium. The plating efficiencies of cells in the presence of various concentrations of drugs were determined either by complete dose–response curves [20, 21] or by a semi-quantitative procedure [14, 18]. For complete dose–response curves, 1 ml volumes of drug (five times the desired final concentration) and cells (between 250 and 500) were added in that order to 60 mm diameter tissue culture dishes containing 3 ml of the medium. The dishes were incubated for 7–12 days at 37° in a 95% O_2–5% CO_2 atmosphere incubator, after which they were stained with 0.5% methylene blue in 50% methanol, and aggregates of 25 or more cells were counted as colonies. The relative plating efficiencies were determined as the ratio of the number of colonies at a certain drug concentration to that obtained in the absence of the drug [20, 21]. In the semi-quantitative procedure, about 200 to 500 cells, in 0.5 ml volume of growth medium, were added to the wells of a 24-well tissue culture dish (Linbro; Flow Laboratories) containing 0.5 ml of the solutions of the drugs at twice the desired final concentration in growth medium [14, 18]. The dishes were incubated for 6–10 days at 37°, after which they were stained by the procedure described above. The effect of drug on cell growth and plating efficiency was recorded on an arbitrary scale: 3+ (normal) > 2+ > 1+ > ± > − (no growth) (see Tables 2 and 3).

Selection of mutants in HeLa cells. Selection of mutants was carried out by procedures similar to those employed earlier [18, 22]. Exponentially growing HeLa cell cultures were treated with 1–2 μg/ml of the mutagen *N*-methyl-*N'*-nitro-nitrosoguanidine (MNNG) for 2 hr. This treatment resulted in about 30–50 per cent cell killing [18]. The mutagen-treated cells were grown for 3 days in non-selective medium to allow time for mutation fixation [22]. The selection of mutants was carried out by plating 5×10^5 cells/100 mm diameter dish, on several dishes in medium containing the appropriate concentrations of the selective agents. The plating efficiencies of the cells at the time of plating were determined by plating a known number of cells in non-selective medium, and mutation frequencies observed were corrected for this [22].

Chemicals. The sources of various chemicals were as follows: ouabain, oleandrin, oligomycin, strophanthidin, digitoxin, and digitoxigenin were purchased from the Sigma Chemical Co., St. Louis, MO; sanguinarine nitrate and suramin were obtained from ICN Pharmaecuticals Inc., Plainview, NY, and the Mobay Chemical Corp., New York, NY, respectively; cassaine was provided by Dr. T. M. Brody of Michigan State University, East Lansing, MI, and ethacrynic acid (Lot No. L-588, 508-00E74) by Dr. O. H. Siegmund of Merck, Sharp & Dohme, Rahway, NJ. Quindonium bromide (*cis*-isomer, Lot U, CN 98, 866-3) and methylquinolizinum bromide (*cis*-isomer, Lot P, CN 99, 194-3) were provided by Dr. Martin L. Black of the Warner–Lambert Co. Ann Arbor, MI.

RESULTS

Selection of HeLa cells mutants resistant to ouabain and cassaine. Figures 2 and 3 show the dose–response curves of HeLa cells towards ouabain and cassaine, respectively. Both ouabain and cassaine were highly toxic to these cells as has been reported earlier [16]. In the case of ouabain, the plating efficiency of cells was reduced to less than 1×10^{-3} at 7×10^{-8} M of the drug, and at 1×10^{-7} M no clonal growth was observed even when 5×10^5 cells were plated on a dish. For cassaine which was about 100-fold less toxic in comparison to ouabain, similar effects on plating efficiencies were observed at correspondingly higher concentrations of the drug.

To select mutants resistant to these drugs, cells were treated with the mutagen MNNG (see Materials and Methods) which increases both the frequency of resistant mutants in the culture, as well as the variety of observable mutant phenotypes [18]. The mutagen-treated cells were subsequently plated in the presence of drug concentrations that kill all the sensitive cells (see Table 1 and Materials and Methods). Under these conditions, a few distinct clones were seen growing in dishes containing either ouabain or cassaine. The frequencies with which such clones were observed in two independent experiments are shown in Table 1. As can be seen, mutants resistant to ouabain and cassaine were obtained with similar frequencies in both the experiments. Furthermore, the frequency of Oua^R mutants in culture was not appreciably affected by a 10-fold increase

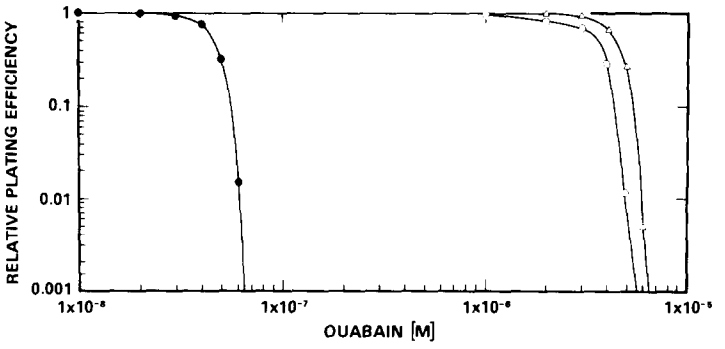

Fig. 2. Dose–response curves showing the cloning efficiencies of sensitive and resistant cell lines in the presence of different concentrations of ouabain. The extrapolation of curves is based upon additional data points not shown here. Key: (●—●) HeLa cells; (○—○) HeLa Oua^R-1 and (△—△) HeLa Oua^R-3.

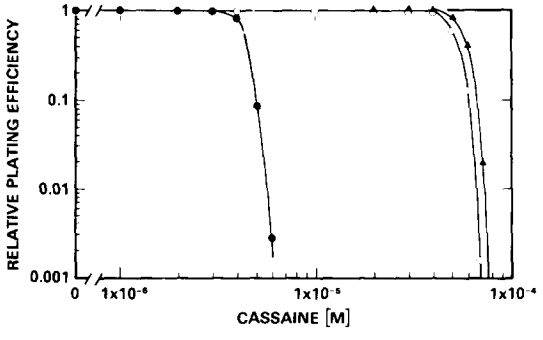

Fig. 3. Survival curves of sensitive and resistant HeLa cell lines in the presence of different concentrations of cassaine. The extrapolation of curves is based upon additional data points not shown here. Key: (●—●) HeLa cells; (○—○) HeLa Cas^R-2; and (▲—▲) HeLa Cas ^{R}-1.

in the selective concentration of ouabain, indicating that clones were highly resistant to ouabain. Several of the clones which appeared in the presence of ouabain and cassaine were picked, and after growth in non-selective medium their degree of resistance towards the selective agent was examined by a semi-quantitative procedure (see Materials and Methods). All of the Oua^R and Cas^R mutants (twelve mutants of each type from the two experiments) which were thus examined were found to be highly resistant to the selective agents. The detailed dose–response curves of two representative Oua^R and Cas^R mutants are given in Figs. 2 and 3; other mutants showed very similar levels of resistance to these drugs. The resistance characteristics of these mutants remained unchanged during growth in non-selective medium for more than 6 months.

Cross resistance pattern of OUA^R and CAS^R mutants. To find out whether mutants separately selected for resistance to ouabain and cassaine exhibited any cross resistance to the other drug, their cross resistance patterns were examined. Results of these studies are shown in Tables 2 and 3. As can be seen, all of the mutants selected for resistance to cassaine showed high levels of cross resistance to ouabain and in this respect were similar to the Oua^R mutants (Table 2). The mutants selected for resistance to ouabain, likewise, showed a high degree of cross resistance to cassaine, similar to that seen with the Cas^R mutants (Table 3), and again no exception was observed.

The cross resistance of Oua^R and Cas^R mutants was also examined against a number of other drugs that have been shown to interact with and inhibit the Na^+, K^+-ATPase activity [see Ref. 2]. As can be seen in Table 4, mutants resistant to ouabain and cassaine did not exhibit any cross resistance towards other inhibitors such as sanguinarine [23, 24], quindonium bromide [25], suramin, oligomycin and ethacrynic acid. In contrast to these inhibitors, mutants resistant to both ouabain and cassaine proved highly cross resistant to several other cardiac glycosides and aglycones (e.g. oleandrin, strophanthidin, digitoxin and digitoxigenin) which were examined, and their cross resistance patterns were identical (Table 5).

Table 1. Frequency of Oua^R and Cas^R mutants in HeLa cells*

Experiment	Selective Agent	Concentration (M)	No. of cells plated	No. of resistant colonies observed	Mutation frequency
I	Ouabain	1×10^{-7}	3×10^6	165	5.5×10^{-5}
I	Ouabain	1×10^{-6}	2.4×10^6	99	4.1×10^{-5}
I	Cassaine	1×10^{-5}	3×10^6	160	5.3×10^{-5}
I	Cassaine	2×10^{-5}	2.4×10^6	113	4.7×10^{-5}
II	Ouabain	5×10^{-7}	2.5×10^6	42	1.7×10^{-5}
II	Cassaine	1.2×10^{-5}	2.5×10^6	46	1.8×10^{-5}

* Selection of mutants from mutagen-treated HeLa cell cultures was carried out as described in Materials and Methods.

Table 2. Cross resistance of Cas^R mutants towards ouabain*

Cell lines	Cell growth at different concentrations of ouabain (μM)										
	0	0.01	0.02	0.05	0.1	0.2	0.5	1.0	2.0	5.0	10.0
HeLa	3+	3+	3+	±	−	−	−	−	−	−	−
HeLa Oua^R-7	3+	3+	3+	3+	3+	3+	3+	3+	3+	2+	±
HeLa Oua^R-8	3+	3+	3+	3+	3+	3+	3+	3+	2+	1+	−
HeLa Cas^R-1	3+	3+	3+	3+	3+	3+	3+	3+	3+	2+	±
HeLa Cas^R-2	3+	3+	3+	3+	3+	3+	3+	3+	2+	1+	−
HeLa Cas^R-3	3+	3+	3+	3+	3+	3+	3+	3+	2+	1+	−
HeLa Cas^R-4	3+	3+	3+	3+	3+	3+	3+	3+	2+	1+	−
HeLa Cas^R-5	3+	3+	3+	3+	3+	3+	3+	3+	3+	2+	±
HeLa Cas^R-7	3+	3+	3+	3+	3+	3+	3+	3+	2+	1+	±
HeLa Cas^R-8	3+	3+	3+	3+	3+	3+	3+	3+	2+	1+	−
HeLa Cas^R-9	3+	3+	3+	3+	3+	3+	3+	3+	2+	1+	−

* The degree of resistance of various cell lines towards ouabain was determined by a semi-quantitative procedure as described in Materials and Methods. The effect of ouabain on the colony-forming ability of cells was assessed on a rough scale of 3+ to −. In this scale, 3+ indicates normal growth similar to that observed in control cells in the absence of any drug; $2+ < 1+ < \pm < -$ indicate increasing effect on cell growth. The values of 1+ in this table roughly corresponded to the D_{10} value of the drug, i.e. concentration of the drug which reduced cloning efficiency of cells to 10 per cent of the untreated control. Cross resistance data are shown for only some of the mutants. The other Oua^R and Cas^R mutants examined showed very similar degrees of resistance.

Table 3. Cross resistance of Oua^R mutants towards cassaine*

Cell lines	Cell growth at different concentrations of cassaine (μM)											
	0	2	4	6	8	10	15	20	30	50	75	100
HeLa	3+	3+	3+	±	−	−	−	−	−	−	−	−
HeLa Cas^R-1	3+	3+	3+	3+	3+	3+	3+	3+	3+	2+	1+	−
HeLa Cas^R-2	3+	3+	3+	3+	3+	3+	3+	3+	2+	1+	±	−
HeLa Oua^R-1	3+	3+	3+	3+	3+	3+	3+	3+	3+	1+	±	−
HeLa Oua^R-3	3+	3+	3+	3+	3+	3+	3+	3+	3+	2+	1+	±
HeLa Oua^R-6	3+	3+	3+	3+	3+	3+	3+	3+	3+	2+	1+	−
HeLa Oua^R-7	3+	3+	3+	3+	3+	3+	3+	3+	3+	1+	±	−
HeLa Oua^R-8	3+	3+	3+	3+	3+	3+	3+	3+	3+	1+	±	−

* The degree of resistance of various cell lines towards cassaine was determined by a semi-quantitative procedure as described in Materials and Methods and Table 2. The cross resistance data are shown for only some representative mutant cell lines. The other Oua^R and Cas^R mutants examined exhibited very similar degrees of resistance to cassaine.

Table 4. Cross resistance studies with other inhibitors of Na^+,K^+-ATPase*

Compounds	Approximate D_{10} values of the cell lines		
	HeLa	HeLa Oua^R	HeLa Cas^R
Ethacrynic acid	5 μg/ml	5 μg/ml	5 μg/ml
Sanguinarine	60 μg/ml	40 μg/ml	40 μg/ml
Suramin	300 μg/ml	300 μg/ml	300 μg/ml
Quindonium bromide (*cis*-isomer)	100 μg/ml	100 μg/ml	100 μg/ml
Methylquinolizinum bromide (*cis*-isomer)	15 μg/ml	15 μg/ml	15 μg/ml
Oligomycin	1 ng/ml	1 ng/ml	1 ng/ml

* The D_{10} values refer to the concentration of the drugs which reduced cloning efficiency of a cell line to 10 per cent of that observed in the absence of any drug. The approximate D_{10} values (range of variation ± 10 per cent) of the cell lines towards various drugs were determined by semi-quantitative methods as described in Tables 2 and 3. A number of Oua^R and Cas^R mutants were examined in these studies, and they all behaved very similarly.

Table 5. Cross resistance of Oua^R and Cas^R mutants to other cardiac glycosides and aglycones*

Compound	Approximate D_{10} values of the cell lines		
	HeLa	HeLa Oua^R-1	HeLa Cas^R-1
Strophanthidin	2×10^{-7} M	$>2 \times 10^{-5}$ M	$>2 \times 10^{-5}$ M
Oleandrin	1.5×10^{-8} M	1×10^{-5} M	8×10^{-6} M
Digitoxigenin	2×10^{-7} M	$>2 \times 10^{-5}$ M	$>2 \times 10^{-5}$ M
Digitoxin	3×10^{-8} M	2×10^{-5} M	1.5×10^{-5} M

* The approximate D_{10} values (range of variation ± 10 per cent) of the cell lines towards various drugs were determined by the semi-quantitative procedure. Representative results with only one Oua^R and one Cas^R mutants are shown here. All the other Oua^R and Cas^R mutants examined behaved very similarly.

DISCUSSION

Results presented in this paper show that, when cellular mutants resistant to ouabain and cassaine are selected from mutagenized HeLa cells, the same types of mutants are obtained with both agents. The two types of mutants appear identical by a number of genetic criteria such as (i) identical mutation frequency in mutagenized cultures in independent experiments (Table 1), (ii) identical patterns and levels of cross resistance to ouabain, cassaine and a number of other cardiac aglycones and glycosides (Tables 2, 3 and 5), and (iii) lack of cross resistance to other inhibitors of Na^+,K^+-ATPase which indicate that cross resistance between ouabain and cassaine is highly specific in nature (Table 4).

Both ouabain and cassaine are known to be specific inhibitors of plasma membrane Na^+,K^+-ATPase and exhibit very similar binding characteristics to this enzyme [8, 9]. Earlier studies have shown that mutants resistant to ouabain in various types of cultured mammalian cells involve alteration in Na^+,K^+-ATPase, which as a result shows altered binding affinity towards cardiac glycosides [15, 16, 26]. If ouabain and cassaine were acting at different sites on this enzyme, then when mutants resistant to ouabain and cassaine were selected from a mutagenized cell population that presumably contains a variety of mutant phenotypes, it would be expected that at least some of the mutants resistant to one drug would not exhibit cross resistance to the other. However, our finding that all of the Oua^R and Cas^R mutants exhibited identical cross resistance patterns to both the drugs, and that no mutant which showed resistance to only one of these drugs was observed, provides strong evidence that both of these drugs interact with the same site on the target enzyme Na^+,K^+-ATPase. In earlier studies, where similar genetic results were obtained with a different group of compounds, its basis was shown to lie in the presence of common structural determinants in these compounds [10, 13]. In this situation, the mutants in each case are selected against the same common determinants and, hence, behave identically [13].

The marked similarity in the biochemical and pharmacological effects of cardiac glycosides and erythrophleum alkaloids has led to the suggestion that the biological activities of these two groups of compounds may be due to common structural determinants and that the structural features which are presently considered essential based on studies with cardiac glycosides, need to be revised [1, 6, 27]. The genetic studies described here strongly support this view. The structure–activity relationships of various cardiac glycosides, erythrophleum alkaloids and other related compounds such as prednisolene and 3,20-bis-(guanylhydrazone) are currently being investigated in a cell culture model system to identify the structural features essential for their activities

The Oua^R and Cas^R mutants of HeLa cells, which presumably are altered at a common binding site for these drugs, do not show any cross resistance to some of the other proposed inhibitors of Na^+, K^+-ATPase, such as ethacrynic acid, sanguinarine, quindonium bromide, suramin and oligomycin, which were examined. These results provide strong evidence that the sites of action of these inhibitors are different from that of cardiac glycosides and erythrophleum alkaloids.

Acknowledgements—I thank Rajni Gupta and Malcolm Moffat for technical assistance in these experiments, Dr. D. R. McCalla for his helpful comments on the manuscript, and Dr. T. M. Brody for providing cassaine. The work was supported by grants from the Medical Research Council of Canada.

REFERENCES

1. I. M. Glynn, *Pharmac. Rev.* **16**, 381 (1964).
2. A. Schwartz, G. E. Lindenmayer and J. C. Allen, *Pharmac. Rev.* **27**, 3 (1975).
3. T. Akera, *Science* **198**, 569 (1977).
4. T. Akera and T. M. Brody, *Pharmac. Rev.* **29**, 187 (1978).
5. C. Fisch and B. Surawicz (Eds.), *Digitalis*, pp. 1–230. Grune & Stratton, New York (1969).
6. R. H. Thorp and L. B. Cobbin, *Cardiac Stimulant Substances*, pp. 137–49. Academic Press, New York (1967).
7. J. B. Kahn, Jr., *Proc. Soc. exp. Biol. Med.* **110**, 412 (1962).
8. T. Tobin, T. Akera, S. L. Brody, D. Ku and T. M. Brody, *Eur. J. Pharmac.* **32**, 133 (1975).
9. T. Tobin, T. Akera, T. M. Brody and H. R. Taneja, *Eur. J. Pharmac.* **35**, 59 (1976).
10. R. S. Gupta and L. Siminovitch, *Biochemistry* **16**, 3209 (1977).
11. R. S. Gupta and L. Siminovitch, *Somat. Cell Genet.* **4**, 355 (1978).

12. R. S. Gupta and L. Siminovitch, *Somat. Cell Genet.* **6**, 151 (1980).
13. R. S. Gupta and L. Siminovitch, *Molec. Pharmac.* **18**, 136 (1980).
14. R. S. Gupta, *Somat. Cell Genet.* **7**, 59 (1981).
15. R. M. Baker, D. M. Brunette, R. Mankowitz, L. H. Thompson, G. F. Whitmore, L. Siminovitch and J. E. Till, *Cell* **1**, 9 (1974).
16. H. M. Rosenberg, *J. cell. Physiol.* **85**, 135 (1975).
17. T. T. Puck, P. I. Marcus and S. J. Cieciura, *J. exp. Med.* **103**, 273 (1956).
18. R. S. Gupta, *Mutation Res.*, in press (1981).
19. R. S. Gupta, *J. cell. Physiol.* **104**, 241 (1980).
20. R. S. Gupta and L. Siminovitch, *Cell* **9**, 213 (1976).
21. R. S. Gupta and L. Siminovitch, *J. cell. Physiol.* **102**, 305 (1980).
22. R. S. Gupta and L. Siminovitch, *Mutation Res.* **69**, 113 (1980).
23. K. D. Straub and P. Carver, *Biochem. biophys. Res. Commun.* **62**, 913 (1975).
24. E. Seifen, R. J. Adams and R. K. Reimer, *Eur. J. Pharmac.* **60**, 373 (1979).
25. H. D. Brown, *Biochem. Pharmac.* **15**, 2007 (1966).
26. W. N. Choy and J. W. Littlefield, *Proc. natn. Acad. Sci. U.S.A.* **77**, 1101 (1980).
27. R. Thomas, J. Boutagy and A. Gelbert, *J. pharm. Sci.* **63**, 1649 (1974).