*Biochemical Pharmacology*, Vol. 34, No. 9, pp. 1501–1505, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00


# INVESTIGATIONS OF THE ACTION OF THE ANTITUMOUR DRUG ADRIAMYCIN ON TUMOUR CELL MEMBRANE FUNCTIONS—I

SURESH B. CHAHWALA and JOHN A. HICKMAN

Cancer Research Campaign Experimental Chemotherapy Group, Department of Pharmacy, University of Aston in Birmingham, Birmingham B4 7ET, U.K.

(*Received* 20 *August* 1984; *accepted* 13 *November* 1984)

**Abstract**—The membrane potential of L1210 murine leukemia cells was assessed by use of the tritiated lipophilic cation probe triphenylmethylphosphonium bromide. The potassium equilibrium potential of the cells was found to be $-71 \pm 7$ mV. The resting membrane potential was partly dissipated by the protonophore *m*-chlorocarbonylcyanidephenylhydrazone (10 μM), but was unaffected by ouabain (1 mM) and apparently by the calcium ionophore A23187 (2.5 μM). Monensin (20 μM) caused a hyperpolarization which, since it was blocked by ouabain, was presumed to be brought about by activation of the $Na^+K^+$-ATPase *via* an elevated cytoplasmic $Na^+$ concentration. Adriamycin at concentrations as high as $5 \times 10^{-4}$ M brought about no change in the resting potential of the cells. Also, cytotoxic concentrations of adriamycin, unlike ouabain, had no effect on rubidium-86 transport into L1210 cells, nor upon a monensin-induced increased in rubidium-86 uptake. The results suggest that although adriamycin is capable of interaction with the plasma membrane, and may exert its cytotoxicity at this locus, changes in ion flux mediated by $Na^+K^+$-ATPase or those capable of changing the membrane potential do not appear to be implicated in its mechanism of action.

The nature of the cellular locus of action of the antineoplastic drug adriamycin is controversial. It is capable of physical interaction with both DNA and membranes [1, 2]. Powerful evidence that its cytotoxicity is expressed through a membrane-mediated effect comes from studies using polymer-immobilized adriamycin, which does not enter the cell. It was shown that the polymer–drug complex was more potently cytotoxic than free drug [3]. Numerous reports have been made of the biophysical effects of adriamycin on cellular or model membranes [2] but few studies have attempted to address the question of what consequences may ensue from an adriamycin–membrane interaction which could account for cytotoxicity. Early changes in the order-parameter of the plasma membrane of murine S180 sarcoma cells, induced by concentrations of adriamycin as low as $10^{-7}$ M and measured by electron spin resonance, were suggestive of profound effects on the function of integral membrane proteins such as sodium–potassium ATPase ($Na^+K^+$-ATPase) which controls alkali metal ion flux [4]. Growth control may be mediated through changes in ion flux [5] and membrane potential [6] and it is possible, therefore, that adriamycin could disrupt these regulatory controls. It is also possible that adriamycin-induced changes in membrane function could alter calcium homeostasis and this may be responsible for cell death. Calcium is cytotoxic if concentrations of above 5 μM are maintained in the cytoplasm [7] and changes in calcium homeostatis in the heart have been implicated in adriamycin-induced cardiac toxicity [8] and, less convincingly, in its cytotoxicity [9]. Changes in cardiac function brought about by adriamycin have also been related to its ouabain like effects, although a claim of the potent inhibition of $Na^+K^+$-ATPase activity by adriamycin [10] has been disputed [11, 12].

We report here the determination and characterization of the membrane potential ($E_m$) of L1210 leukemia cells using the lipophilic cation probe $TPMP^+$ and the effects of adriamycin on this potential. Phosphonium probes have been used in studies of membrane potential changes occurring in biological events mediated at this level of the membrane such as mitogenesis [13] and receptor ligand binding [14]. The membrane potential of the cell reflects the consequences of the complex traffic of ions across cellular membranes, and we considered that any perturbation of this ion traffic by adriamycin may be reflected in changes of potential. We have also attempted to determine whether the activity of $Na^+K^+$-ATPase is affected by adriamycin, as predicted from the early-induced changes in order parameter brought about by low concentrations of drug [4].

## MATERIALS AND METHODS

*Materials.* Analytical grade chemicals were obtained from BDH Ltd. (Poole, U.K.) unless otherwise stated. Ouabain octahydrate, monensin and *m*-chlorocarbonylcyanidephenylhydrazone (CCCP)* were obtained from Sigma Chemical Company (London, U.K.). [$^3$H]$H_2O$ (5 mCi/ml) [$^3$H]PEG (1.6 mCi/g) and rubidium-86 chloride (1.77 μCi/

* Abbreviations used: $TPMP^+$, triphenylmethylphosphonium bromide; CCCP, *m*-chlorocarbonylcyanidephenylhydrazone; PEG, polyethylene glycol; $^{86}$Rb, rubidium-86.

1.17 mg) from the Radiochemical Center (Amersham, U.K.). [$^3$H]TPMP$^-$ (41.5 Ci/mmole) and NEN 260 scintillation cocktail were obtained from New England Nuclear (Southampton, U.K.). Unlabelled TPMP$^+$ was purchased from K & K Laboratories (via Kodak, Liverpool). Adriamycin and amiloride were gifts from Farmitalia (Milan, Italy) and Merck Sharp and Dohme Ltd. (Hoddesdon, U.K.) respectively. Media and serum were obtained from Gibco Ltd. (Glasgow, U.K.).

*Cell culture.* Murine L1210 leukemic cells obtained from Flow Laboratories (Glasgow) were maintained as a suspension culture in RPMI 1640 medium with 10% horse serum. The cells were cultured at 37° in 90% air 10% $CO_2$ with a doubling time of 12–14 hr. Experiments were performed with cells in mid to late log phase.

*Assays of cytotoxicity.* $2 \times 10^4$ L1210 cells/ml in log phase were incubated with various concentrations of adriamycin for 1 and 2 hr. The cells were centrifuged at 600 *g*, washed once with Dulbecco's phosphate buffered saline (pH 7.4), and resuspended in RPMI 1640 media with 10% horse serum, incubated at 37° and counted 3 days later. The inhibitory concentration to prevent 90% of the increase in cell number observed in the controls was estimated. During the course of incubation of L1210 cells with ionophores and various drugs viability was determined by estimation of exclusion of a solution of 0.1% Trypan Blue dye in saline.

*Estimation of L1210 membrane potentials.* Cells were harvested and washed in either high or low concentration solutions of potassium (see below) and 100 $\mu$l of a suspension of $2 \times 10^6$ washed cells was added to 900 $\mu$l of solutions containing either a high or low concentration of potassium at 37° which were gassed with 95% air 5% $CO_2$. The low potassium solution contained 118 mM NaCl, 5 mM KCl, 1.27 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.18 mM $MgSO_4$, 1.17 mM $KH_2PO_4$ and 5.5 mM glucose (pH 7.4). The high potassium solution contained 123 mM KCl, 1.27 mM $CaCl_2$, 25 mM $KHCO_3$, 1.18 mM $MgSO_4$, 1.17 mM $KH_2PO_4$ and 5.5 mM glucose (pH 7.4). These solutions contained 2–30 $\mu$M of [$^3$H]TPMP$^-$ (0.2–3 $\mu$Ci/ml). After certain times (see results) of incubation at 37°, with continuous gassing, 200 $\mu$l aliquots were removed in triplicate and centrifuged at 9000 *g* for 1 min in a Beckmann microfuge through a mixture of 5 parts of Dow-Corning 550 silicon oil with 1.5 parts of corn oil (Mazola), which was layered on top of 50 $\mu$l of 98% formic acid. The centrifuge tubes were then frozen in liquid nitrogen and cut through the oil layer. The pellet and supernatant were placed separately into glass vials, thawed, NEN 260 scintillation cocktail added and counted on a Packard Tricarb 2606 scintillation counter. In each of these assays intracellular volume was calculated by determination of the volume of the pellet with [$^3$H]$H_2O$ and extracellular space with [$^3$H]PEG [15].

*Calculation of $E_m$ from [$^3$H]TPMP$^-$ distribution.* The concentration of [$^3$H]TPMP$^+$ accumulated by the cells was calculated by using values for intracellular volume and $E_m$ calculated as follows [15]:

$$E_m = 2.3\,RT \log [\mathrm{TPMP}^+]_{in}^{\text{corrected}} / [\mathrm{TPMP}^+]_{out}$$

in which

$2.3\,RT$ is equal to $-61$ mV at 37°

and

$$[\mathrm{TPMP}^+]_{in}^{\text{corrected}} = [\mathrm{TPMP}^+]_{in}^{\text{low K}^+} - [\mathrm{TPMP}^+]_{in}^{\text{high K}^+}.$$

*Effects on rubidium-86 uptake.* L1210 cells were washed and suspended at $2 \times 10^6$/ml in low K$^+$ buffer and then added to the same buffer containing 5 $\mu$Ci/ml $^{86}$Rb with or without drugs. The cells were incubated at 37° with continuous gassing for given times and aliquots centrifuged and processed, as described above. $^{86}$Rb was counted on an ICN Tracerlab, Gamma Set 500 gamma counter.

## RESULTS

In suspension culture the $ID_{90}$ for adriamycin incubated for 1 hr with L1210 cells was $2.6 \times 10^{-7}$ M. The effects of $5 \times 10^{-4}$ M adriamycin on membrane potential were also investigated as this concentration

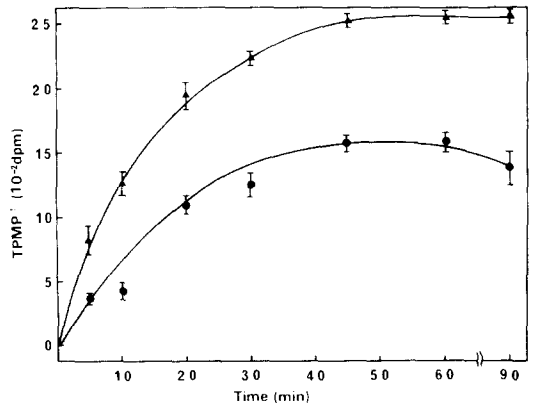


Fig. 1. Accumulation, with time, of the lipophilic cation [$^3$H]TPMP$^+$ into L1210 cells. Cells at $2 \times 10^6$/ml were incubated in high and low K$^+$ medium with 2 $\mu$M [$^3$H]TPMP$^+$. Samples were removed at intervals and processed as described in Materials and Methods: high K$^+$ (●) and low K$^+$ (▲) medium. A typical result with each point representing the mean of triplicate determinations.

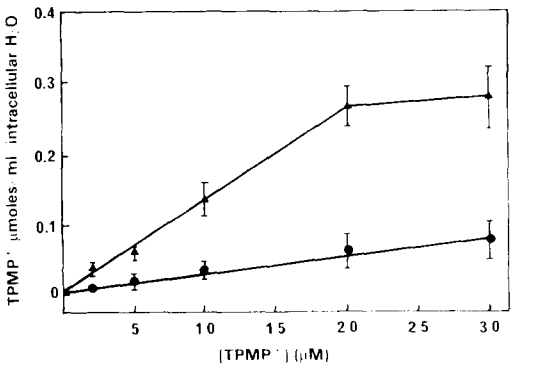


Fig. 2. Accumulation of [$^3$H]TPMP$^+$ in L1210 cells after 1 hr incubation in high and low K$^+$ at various [$^3$H]TPMP$^+$ concentrations. Cells at $2 \times 10^6$/ml were incubated with 2–30 $\mu$M [$^3$H]TPMP$^+$ in high and low K$^+$ for 1 hr at 37°: high K$^+$ (●) and low K$^+$ (▲) medium. Each point represents the mean of two experiments assayed in triplicate.

was previously shown to affect ion transport in bladder epithelia [16].

The time course of the accumulation of 2 $\mu$M [$^3$H]-TPMP$^+$ is shown in Fig. 1. The accumulation in L1210 cells incubated in low $K^+$ medium, equivalent to physiological concentration, was rapid for approximately 20 min and then reached a plateau phase, reaching steady-state by 45 min. In high $K^+$ medium both the initial rate of accumulation and the level of the steady state accumulation were depressed. Figure 2 shows that concentrations from 2 to 20 $\mu$M of [$^3$H]TPMP$^+$ were accumulated linearly, a process suggestive of passive equilibration with the membrane potential via a non-saturatable process. At the low potassium concentration the accumulation of [$^3$H]TPMP$^+$ reached steady state equilibrium, whereas at a high potassium concentration accumulation continued to be linear up to 30 $\mu$M [$^3$H]TPMP$^+$.

The potassium equilibrium potential of L1210 cells ($E_m$) was calculated by subtracting the accumulation of [$^3$H]TPMP$^+$ in high potassium medium from that in low potassium medium, as described above, and entering the concentration of the lipophilic cation present at equilibrium into the Nernst equation. In experiments which measured the time taken to reach equilibrium (Fig. 3), the potassium equilibrium potential at 45 min was calculated to be $-74 \pm 4$ mV (N = 3). Data accumulated at a single 60-min time point gave a value of $71 \pm 7$ mV (N = 14) (Table 1). [$^3$H]TPMP$^+$ had no effect on cell viability as measured by exclusion of trypan blue.

The sodium–proton exchanger/ionophore monensin (20 $\mu$M) induced a profound hyperpolarization of L1210 cells as reflected by increased accumulation of [$^3$H]TPMP$^+$ (Table 1). This hyperpolarization was blocked by coincubation with 1 mM ouabain, strongly suggesting that, in common with other studies [17], the monensin-induced sodium influx activates $Na^+K^+$-ATPase. This was confirmed by the observation of a ouabain-sensitive increase in $^{86}$Rb uptake after monensin (Fig. 4). This enhanced $^{86}$Rb uptake was completely unaffected in cells co-incubated with adriamycin $2.6 \times 10^{-7}$ M for 1 hr (Fig. 4), a concentration which kills 90% of the cells, and at which Murphree *et al.* [4] had shown changes in the order parameter of tumour cell membranes.

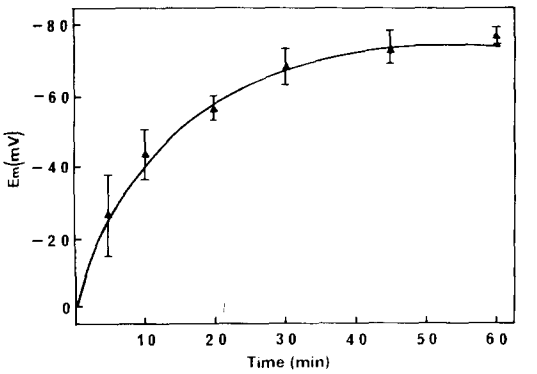


Fig. 3. The potassium equilibrium potential of L1210 cells at 37°. The equilibrium potential of L1210 cells ($E_m$) was calculated by subtracting accumulation of 2 $\mu$M [$^3$H]TPMP$^+$ in high $K^+$ from that in low $K^+$ and by entering the concentrations of [$^3$H]TPMP$^+$ at equilibrium into the Nernst equation. Each point represents the mean ± S.E. of three experiments.

Figure 5 shows the effects of ouabain (1 mM) and adriamycin ($2.6 \times 10^{-7}$ M) on $^{86}$Rb uptake over 1 hr into L1210 cells. Ouabain had a profound effect ($P = <0.02$) on the initial rate of uptake whereas adriamycin had an insignificant effect ($P = <0.05$) at this concentration or at $5 \times 10^{-4}$ M (data not shown).

The protonophore CCCP (10 $\mu$M) significantly decrease the accumulation of the lipophilic cation under conditions of both high and low potassium indicating depolarization. The former result suggests that in cells equilibrated with potassium [$^3$H]TPMP$^+$ accumulation may be related to the mitochondrial membrane potential generated by a proton gradient in a manner similar to that suggested for neuroblastoma-glioma hybrid cells [15].

In order to assess what effect changes in intracellular $Ca^{2+}$ have on L1210 cell membrane potential, the cells were incubated with the calcium ionophore A23187 (2.5 $\mu$M). At this concentration of A23187 there was no change in the cell viability. The lack of effect on $E_m$ (Table 1) is a somewhat surprising result given that normal mouse lymphocytes were observed to hyperpolarize after induction of a calcium influx [18] and the result is discussed in detail below. At concentrations of A23187 above

Table 1. Membrane potentials ($E_m$) of L1210 cells incubated at 37° for 1 hr with various membrane-active agents

| Treatment | $E_m$ (mV)* | % Control |
|---|---|---|
| Control | $-71 \pm 7$ | 100 (N.S.)† |
| Adriamycin ($5 \times 10^{-4}$ M) | $-72 \pm 16$ | 101 (N.S.) |
| Ouabain (1 mM) | $-61 \pm 2$ | 86 (N.S.) |
| Monensin (20 $\mu$M) | $-91 \pm 1$ | 128 ($P < 0.025$) |
| Monensin + ouabain | $-75 \pm 5$ | 106 (N.S.) |
| A23187 (2.5 $\mu$M) | $-70 \pm 10$ | 99 (N.S.) |
| CCCP (10 $\mu$M) | $-25 \pm 13$ | 35 ($P < 0.02$) |

L1210 cells at $2 \times 10^6$/ml were incubated at 37° for 1 hr with 2 $\mu$M [$^3$H]-TPMP$^+$ in high and low $K^+$ in presence of the indicated drugs. $E_m$ was calculated using the Nernst equation as described in Materials and Methods.

* Membrane potential for control represents the mean ± S.E. of 14 independent experiments. For the rest it is mean ± S.E. of at least 3 experiments.

† $P > 0.05$ was judged to be not significant (N.S.).

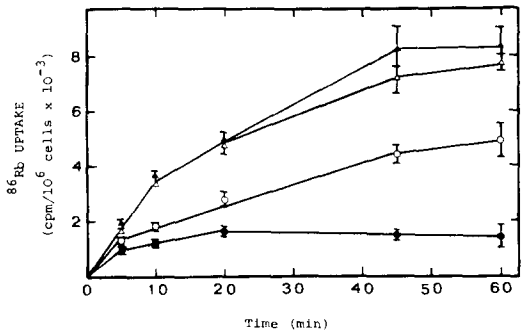


Fig. 4. The effects of ouabain and adriamycin on monensin induced $^{86}$Rb uptake into L1210 cells. Cells at $2 \times 10^6$/ml were coincubated at 37° at time zero with $^{86}$Rb (5 $\mu$Ci/ml) with and without drugs: ○, control; ▲, 20 $\mu$M monensin; △, 20 M monensin + $2.6 \times 10^{-7}$ M adriamycin; ●, 20 $\mu$M monensin + 1 mM ouabain. A typical result with each point representing the mean of triplicate determinations.

2.5 $\mu$M loss of cell viability was observed. Adriamycin, after a 1 hr incubation at concentrations of $2.6 \times 10^{-7}$ M and up to $5 \times 10^{-4}$ M had no significant effect on the membrane potential of L1210 cells (Table 1). Ouabain (1 mM) was also without effect on [$^3$H]TPMP$^+$ accumulation, suggesting that the sodium pump contributes little to the maintenance of the resting membrane potential of L1210 cells.

## DISCUSSION

The plasma membrane of tumour cells represents to the experimental chemotherapist an attractive locus for the targeting of drugs. It is here that many of the functions which control cell proliferation are expressed, and which are aberrant in the neoplastic state. We are attempting to establish whether the well documented effects of the cytotoxic drug adriamycin on the physical properties of membranes may lead to cell death through the disruption of important events occurring at the level of the membrane and in the present study we address those involving ion flux, as reflected in changes in membrane potential and the activity of $Na^+K^+$-ATPase. The characterization of the potential and of those ion fluxes

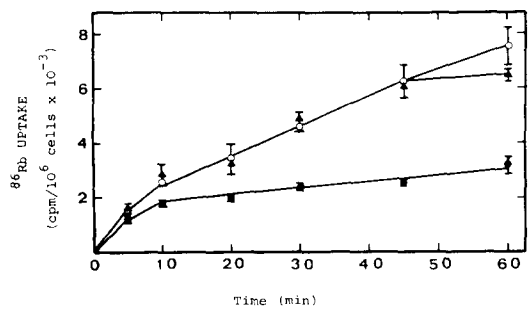


Fig. 5. The effects of ouabain and adriamycin on $^{86}$Rb uptake into L1210 cells. Cells at $2 \times 10^6$/ml were coincubated at 37° at time zero with $^{86}$Rb (5 $\mu$Ci/ml) with and without drugs: ○, control; ▲, $2.6 \times 10^{-7}$ M adriamycin; ●, 1 mM ouabain. A typical result with each point representing the mean of triplicate determinations.

responsible for it in L1210 cells is thus important in such a study.

The profiles of accumulation of [$^3$H]TPMP$^+$ by L1210 leukemia cells (Figs. 1 and 2) are similar to those observed for murine spleen lymphocytes [13], and this accumulation is accomplished in the same way by a passive transport of the probe (Fig. 2). As with the normal lymphocytes, accumulation is inhibited by about 50% when the cells are incubated in a high concentration of extracellular potassium (Fig. 2), presumably because they are depolarized to the extent of the potassium equilibrium potential. The residual accumulation of [$^3$H]TPMP$^+$ observed at a high potassium concentration may represent a multitude of mechanisms whereby the cell continues to take up the cation. In particular there is the contribution of organelle potentials such as the mitochondria, the contribution of electrogenic equilibria other than the potassium dependent potential, which are generated at the plasma membrane, such as proton equilibria [19], and finally non-specific binding.

Estimation of the potassium equilibrium potential of L1210 cells by the method outlined above gave a value of $-71 \pm 7$ mV (N = 14). This is slightly higher than values obtained for normal mouse spleen lymphocytes obtained by this method ($-65 \pm 2$ mV) [13] or by the use of fluorescent dyes ($-60$ mV) [18]. In a study of normal and transformed hamster lymphocytes the malignant counterparts had a lowered membrane potential [20].

Ouabain was found to be without effect on the resting membrane potential of L1210 cells and it thus appears that electrogenic pumping of ions by $Na^+K^+$-ATPase makes little contribution to the resting potential. This lack of effect was shown not to be the result of ouabain insensitivity of these murine cells, a common problem, since the uptake of $^{86}$Rb was inhibited under the same conditions (Fig. 5). Elevation of intracellular sodium by use of the ionophore monensin, which exchanges $Na^+$ for $H^+$, is known to activate $N^+K^+$-ATPase [17] and indeed the hyperpolarisation observed here was blocked by ouabain (Table 1). Adriamycin at its $ID_{90}$ to L1210 cells had no effect on $^{86}$Rb transport nor upon the monensin-induced increase in $^{86}$Rb transport (Fig. 4), strongly suggesting that $Na^+K^+$-ATPase activity is not a target for the drug. This latter result is a finding similar to that of Landolph *et al.* who showed adriamycin to have minimal activity against the $Na^+K^+$-ATPase of C3H/10T½ cells at concentrations which were cytotoxic [21].

Adriamycin alone, at concentrations up to $5 \times 10^{-4}$ M for 1 hr had no significant effects on the resting membrane potential of L1210 cells (Table 1). If, as in the heart, calcium concentrations are increased by adriamycin it might be expected that, as in normal lymphocytes, the cells would hyperpolarize as potassium efflux is activated [18]. However, the calcium ionophore A23187 also apparently had no effect on the resting membrane potential (Table 1). Further studies suggest that indeed there is a hyperpolarization induced by A23187 but that it is not easily detected by changes in [$^3$H]TPMP$^+$ accumulation as it is accompanied by a population of cells undergoing depolarization so that the overall

population averaged change in potential appears to be minimal [22]. In preliminary experiments using alternative techniques capable of detecting A23187-induced changes in $E_m$, through altered $Ca^{2+}$ homeostasis, adriamycin also had no effect.

In conclusion, although adriamycin has been reported to interact with plasma membranes and produces early changes in membrane properties (<1 hr) at concentrations within the cytotoxic dose range used here [4] and the range achieved clinically [23] there appears to be no early, profound effect on membrane permeability as reflected in changes of membrane potential. The present studies also support previous results which showed that the drug does not inhibit $Na^+K^+$-ATPase activity [11, 21]. It therefore remains to determine what specific effects, if any, are exerted by the drug–membrane interaction and how they relate to cytotoxicity. The observation of the lack of effects of adriamycin at cytotoxic concentrations on $Na^+K^+$-ATPase and membrane potential are of positive value inasfar as this suggests that changes in rather general phenomena, such as order parameter, do not necessarily have general consequence, and that an agent bringing about these changes may interact with the membrane in subtle ways to bring about specific effects. Thus, further studies are needed to identify these effects.

*Acknowledgements*—We wish to acknowledge the generous financial support of the Cancer Research Campaign for these studies.

## REFERENCES

1. F. Arcamone, Doxorubicin, Anticancer antibiotics, in *Medicinal Chemistry*, Vol. 17, Academic Press, London.
2. T. R. Tritton and J. A. Hickman, Cell surface membranes as a chemotherapeutic target, in *Chemotherapy*, Vol. 2 (Ed. F. M. Muggia) pp. 81–131. Martinus Nijhoff, The Hague (1985).
3. T. R. Tritton and G. Yee, *Science* **217**, 248 (1982).
4. S. A. Murphree, T. R. Tritton, P. L. Smith and A. C. Sartorelli, *Biochim. biophys. Acta.* **649**, 317 (1981).
5. A. L. Boynton, W. L. McKeehan and J. F. Whitfield (Eds.), in *Ions, Cell Proliferation and Cancer*, Academic Press, London (1982).
6. J. Boonstra, C. L. Mummery, J. J. van Zoelen, P. T. van der Saag and S. W. de Laat, *Anticancer Res.* **2**, 265 (1982).
7. J. C. Metcalfe, T. Pozzan, G. A. Smith and T. R. Hesketh, *Biochem. Soc. Symp.* **45**, 1 (1980).
8. H. M. Olson, D. M. Young, D. J. Prieur, A. F. LeRoy and R. L. Reagan, *Am. J. Pathol.* **77**, 439 (1974).
9. T. Dasdia, A. DiMarco, M. Groffredi, A. Minghetti and A. Necco, *Pharm. Res. Commun.* **11**, 19 (1979).
10. M. Gosalvez, G. D. V. van Rossum and M. F. Blanco, *Cancer Res.* **39**, 257 (1979).
11. D-H. Kim, T. Akera and T. M. Brodie, *J. Pharmac. exp. Ther.* **214**, 368 (1980).
12. L. P. Solomson and P. R. Halabrin, *Cancer Res.* **41**, 570 (1981).
13. H. Kiefer, A. J. Blume and H. R. Kaback, *Proc. natn. Acac. Sci. U.S.A.* **7**, 2200 (1980).
14. J. D-E. Young, J. C. Unkeless, H. R. Kaback and Z. A. Cohn, *Proc. natn. Acad. Sci. U.S.A.* **80**, 1357 (1983).
15. D. Lichtshtein, H. R. Kaback and A. J. Blume, *Proc. natn. Acad. Sci. U.S.A.* **76**, 650 (1979).
16. T. N. Solie and C. Yuncker, *Life Sci.* **22**, 1907 (1978).
17. W. H. Moolenar, C. L. Mummery, P. T. van der Saag and S. W. de Laat, *Cell* **23**, 789 (1981).
18. T. J. Rink, C. Montecucco, T. R. Hesketh and R. Y. Tsien, *Biochim. biophys. Acta* **595**, 15 (1980).
19. A. Heinz, G. Sachs and J. A. Schafer, *J. Membrane Biol.* **61**, 143 (1981).
20. R. B. Mikkelsen and B. Koch, *Cancer Res.* **41**, 209 (1981).
21. J. R. Landolph, R. S. Bhatt, N. Telfer and C. Heidelberger, *Cancer Res.* **40**, 4851 (1980).
22. J. A. Hickman, O. C. Blair, A. L. Stepanowski and A. C. Sartorelli, *Proc. Am. Soc. Cancer Res.* **25**, 13 (1984).
23. R. F. Greene, J. M. Collins, J. F. Jenkins, J. L. Speyer and C. E. Myers, *Cancer Res.* **43**, 3417 (1983).