*Biochemical Pharmacology*, Vol. 32, No. 14, pp. 2189–2194, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00


# ELLIPTICINE-INDUCED ALTERATION OF MODEL AND NATURAL MEMBRANES

FRANÇOIS TERCE, JEAN-FRANÇOIS TOCANNE and GILBERT LANEELLE
Université Paul Sabatier and Centre de Recherche de Biochimie et de Génétique Cellulaires du C.N.R.S., 118 route de Narbonne, 31062 Toulouse Cédex, France

(*Received* 22 *December* 1982; *accepted* 11 *February* 1983)

**Abstract**—The modification of certain membrane properties by ellipticine derivatives was examined. The amphiphilic 9-methoxyellipticine was the most efficient in disorganizing membrane structure and in inducing leaks of liposomes, haemolysis of intact human erythrocytes. This drug and the dipolar 9-amino- and 9-hydroxyellipticine were equally efficient in decreasing the surface charge of membranes and in inhibiting the respiration of an isolated bacterial membrane.

Ellipticine [5,11-dimethyl-6H-pyrido(4,3-b)-carbazole] and its derivatives (Fig. 1) are cytotoxic compounds, some of which have been studied experimentally in cancer chemotherapy [1, 2]. For some time it was considered that their exclusive target was DNA, because they intercalate between bases. This statement was questioned [2, 3] and possible additional targets were investigated. Our group had shown that some ellipticines strongly interact with model and natural membranes containing negatively-charged lipids [4–6]. Physicochemical techniques have shown that these drugs achieve charge neutralization of anionic phospholipids like phosphatidylglycerol [5, 6]. By studying the quenching of fluorescent probes by ellipticine derivatives, we have shown that the more amphiphilic molecules like ellipticine and 9-methoxyellipticine penetrate into model and natural membranes, at least down to the first methylene groups of the phospholipid acyl chains, while dipolar compounds like 9-amino- and 9-hydroxyellipticine interact more superficially (unpublished results). These electrostatic and hydrophobic interactions disturb membranes in their enzymatic functions, or in their role as permeability barriers.

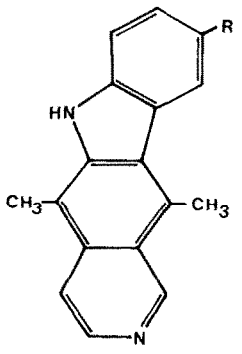


Fig. 1. Ellipticine [5,11-dimethyl-6H-pyrido(4,3b)-carbazole] and some derivatives. R = H, ellipticine; R = OH, 9-hydroxyellipticine; R = $OCH_3$, 9-methoxyellipticine; R = $NH_2$, 9-aminoellipticine.

The effects on permeability were tested on liposomes, isolated erythrocyte membrane and intact human erythrocytes. The inhibition of respiration of isolated bacterial membrane was also followed.

## MATERIALS AND METHODS

### *Chemicals*

Ellipticine derivatives were synthesized and purified either by P. Lecointe (Centre de Pharmacologie et Toxicologie Fondamentales, Toulouse) or P. Lescot (Institut Gustave Roussy, Villejuif).

Egg phosphatidylcholine was prepared according to Lees [7] and purified by preparative high performance liquid chromatography. Bovine brain phosphatidylserine was purchased from Sigma Chemical Co. 6-Carboxyfluoresceine was purchased from Eastman. The purity of these compounds was checked by thin-layer chromatography.

### *Membrane preparations*

*Bacterial membrane.* The membrane of *Micrococcus luteus* (ATCC 4698) was prepared as previously described [8].

*Erythrocyte membrane.* For encapsulation of 6-carboxylfuoresceine, membranes were prepared according to Lepke and Passow [9]. Human erythrocytes, washed twice in 10 mM barbital, 0.9% NaCl, pH 7.2, were diluted 10 times with 1 mM $MgCl_2$ pH 6, at 0° and centrifuged. The pellet was resuspended in 10 mM barbital, 150 mM 6-carboxyfluoresceine pH 7.2, and incubated for 45 min at 40°. The resealed ghosts were sedimented, resuspended in 10 mM barbital, 150 mM NaCl, pH 7.2, and dialysed for 4 hr at 20° against the same buffer. The free 6-carboxyfluoresceine was removed on a Sephadex G 25 column.

*Liposomes.* The liposomes used in measurements of electrophoretic mobility were prepared by vortex dispersion of a mixture of phosphatidylserine and phosphatidylcholine (4:1 molar ratio) in 5 mM phosphate, 45 mM NaCl pH 7.2.

The liposomes used in the permeability assays were prepared by vortex dispersion of 1:1 (molar

ratio) mixtures of phosphatidylserine and phosphatidylcholine in 10 mM barbital, 150 mM carboxyfluoresceine, pH 7.2. Then the mixture was passed twice through a French Press cell and dialysed 1 hr at 20° against 10 mM barbital, 150 mM NaCl, pH 7.2. The free 6-carboxyfluoresceine was removed on a Sephadex G 25 column.

*Electrophoretic mobility*. This was determined by free microelectrophoresis in a Mark II Rank apparatus. Measurements were performed in a glass tube (1.8 mm internal diameter) dipped into a water bath maintained at 25°. Applied tensions were chosen between 5 and 8 V/cm, depending on the experiment. The migration rate of the particles was determined five times in each direction, and the assays were repeated in two independent experiments.

*Leakage of 6-carboxyfluoresceine*. 6-Carboxyfluoresceine encapsulated at a concentration over 100 mM has its fluorescence almost completely quenched. Leakage was followed by measuring the fluorescence of 6-carboxyfluoresceine (excitation wavelength 490 nm, emission wavelength 520 nm) with a Jobin-Yvon J.Y. 3D spectrofluorimeter. Liposomes or released erythrocyte ghosts were diluted in 10 mM barbital, 150 mM NaCl, pH 7.2, at 25°. Complete leakage was obtained by 0.1% Triton X-100 and the fluorescence intensities were normalized by taking the intensity in the presence of Triton to be 100%. The reported values were obtained 6 min after addition of the drugs. Each point represents the average of five determinations.

*Fluorescence polarization*. Isolated membranes (0.02–0.03 mg protein/ml) were incubated overnight at 4° in 45 mM Na, 5 mM phosphate, $2 \times 10^{-6}$ M diphenylhexatriene, pH 7.2, and 30 min at 20° just before the assays. The probe was excited by non-polarized light at 360 nm, and the emitted light was measured at 430 nm The polarization rate is defined as

$$p = (I_V - I_H)/(I_V + I_H)$$

where $I_V$ and $I_H$ are the emission intensities in the vertical and in the horizontal planes. Each reported value is the average of ten measurements. Measurements were performed with a Monnerie and Neel-PF1 apparatus (Magot, France), giving $I_V$ and $I_H$ values that did not need to be corrected.

*Haemolysis*. This was followed with fresh human erythrocytes, washed twice with 10 mM barbital, 0.9% NaCl, pH 7.2. Haemolysis in isotonic medium was followed by incubating erythrocytes for 20 min at 20° in the washing medium with or without addition of drugs. The cells were centrifuged for 2 min at 20°, and the absorbance of the supernatant was measured at 540 nm. Absorbances were normalized by reference to complete haemolysis in water (100% haemolysis).

The anti-haemolytic effects of the drugs were studied by the method of Machleidt *et al.* [10]. Washed erythrocytes were diluted in 1 ml of the isotonic buffer, with $10^{-7}$–$10^{-4}$ M drugs, or without any drug. Barbital (10 mM, pH 7.2) was added in order to obtain after 10 min incubation 20–30% lysis without drug. Haemolysis was measured by the absorbance at 540 nm cell suspension supernatant.

The absorbance of assays without drug was taken as the reference value of 1. Values below 1 indicated a protecting affect of the tested drugs.

*Respiration of the bacterial membrane*. As previously described [8], the substrate was 20 mM L-malate. Oxygen concentrations were determined with an oxygen electrode (YSI) in a 1.4 ml temperature-controlled cell (25°).

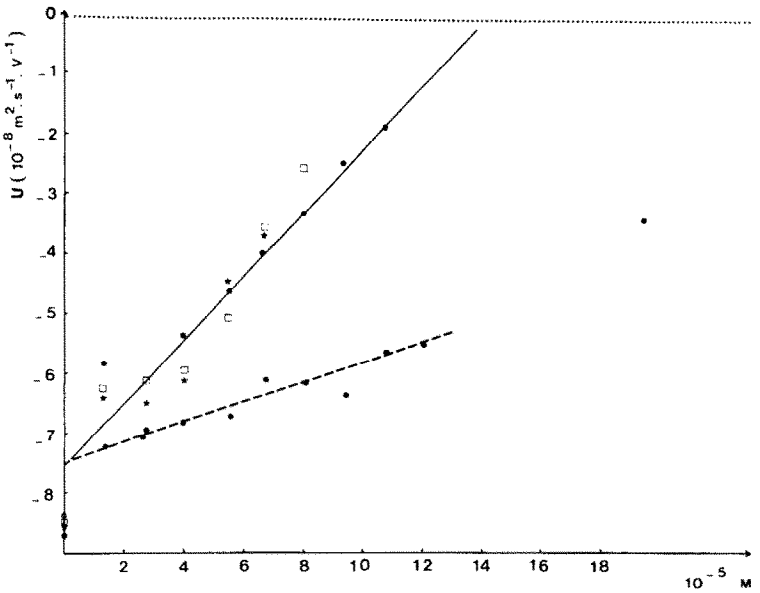


Fig. 2. Electrophoretic mobility of liposomes and isolated bacterial membrane: effect of three ellipticine derivatives. Liposomes and membrane were added to water at 25°. (●) 9-methoxy-, (□) 9-hydroxy-, (☆) 9-aminoellipticine. ——: Liposomes (phosphatidylserine and phosphatidylcholine mixture 4:1, $1.25 \times 10^{-4}$ M total lipids) ----: Membrane isolated from *M. luteus* (0.03 mg protein/ml).

## RESULTS AND DISCUSSION

### *Modification of the electric charge and of membrane organization*

In Fig. 2, the effects of ellipticines on the electrophoretic mobility of membranes, i.e. on their surface charge, are shown.

With liposomes prepared with a mixture of phosphatidylserine and phosphatidylcholine (solid line) the electrophoretic mobility can be lowered almost down to zero, indicating nearly complete neutralization of the surface charge. It is interesting to note that the amphiphilic 9-methoxyellipticine, and the dipolar 9-hydroxy- and 9-aminoellipticine had the same efficiency in this test, while they displayed clear-cut differences when their interaction with monolayers was studied [4, 5], and when their ability to quench fluorescent probes embedded in membranes was considered (unpublished results).

There was also a reduction of the electrophoretic mobility with the membrane isolated from *Micrococcus luteus*, but the membrane precipitated before zero mobility was reached.

Fixation of ellipticines on membranes is also accompanied by a hydrophobic interaction [5, 6]. Both the ionic and the hydrophobic effects are able to change the organization state of membranes. This is seen in Fig. 3, which shows the effect of the three drugs on the fluorescence polarization of diphenylhexatriene embedded in membranes isolated either from human erythrocytes or from *M. Luteus*.

Increasing concentrations of 9-methoxyellipticine lowered the polarization rate of diphenylhexatriene. This depolarization revealed an increased mobility of the probe due to the disordering effect of the drug on both types of membrane.

With 9-amino- and 9-hydroxyellipticine, there was not a significant variation of the polarization rate of diphenylhexatriene.

### *Effect on passive membrane permeability*

The lowering of the electrostatic barrier, and the disordering of the structure are able to impair the passive permeability of membranes. It is clear from Fig. 4 that 9-methoxyellipticine, and to a lesser extent 9-hydroxyellipticine, increased the permeability of liposomes to trapped 6-carboxyfluoresceine. The same effect can be observed with 6-carboxyfluoresceine encapsulated in resealed erythrocyte membrane (Fig. 5).

The permeability increase induced by the ellipticines is also observed with isolated human erythroctyes. In Fig. 6 are reported the percentages of total haemolysis obtained after 20 min in an isotonic medium, in the presence of various drug concentrations. Even in this isotonic medium haemolysis can be detected with $2 \times 10^{-5}$ M 9-methoxyellipticine and with $5 \times 10^{-5}$ M 9-amino- or 9-hydroxyellipticine.

Figure 7 shows the effect of drugs added to erythrocyte preparations which would otherwise have undergone 20–30% total haemolysis. Such lytic assays have been performed with various anaesthetics [11]. Chlorpromazine was tested in our assays as a reference substance. It appears that $10^{-5}$ M 9-methoxyellipticine afforded protection against haemolysis, while in the presence of $2 \times 10^{-5}$ M 9-methoxyellipticine complete lysis occurred. This mimics the effect of chlorpromazine, but 9-methoxyellipticine gave these effects for concentrations five times lower than the corresponding concentrations of chlorpromazine. This corroborates fluorescent probe quenching observations in liposomes (unpublished results), suggesting that the interaction with model membranes was weaker for chlorpromazine than it was for 9-methoxyellipticine.

The effects of 9-aminoellipticine, 9-hydroxyellipticine and chlorpromazine were observed at similar

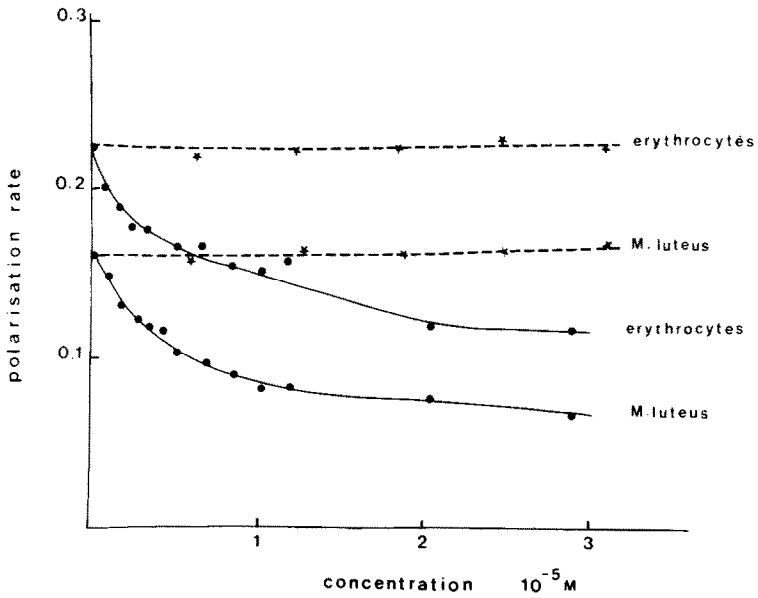


Fig. 3. Effect on the fluorescence polarization rate of diphenylhexatriene inserted in membranes isolated from human erythrocytes and from *M. luteus*. ————: 9-Methoxyellipticine; . . . . : 9-amino- and 9-hydroxyellipticine.

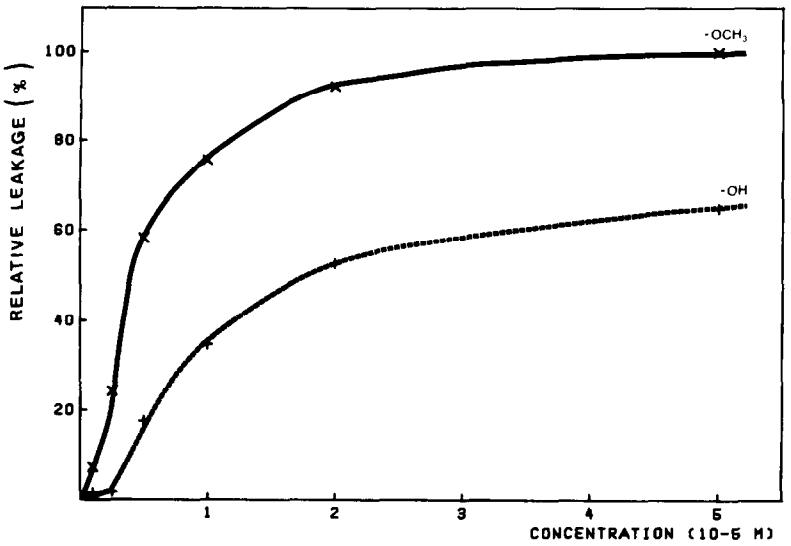


Fig. 4. Leakage of 6-carboxyfluoresceine encapsulated in liposomes: effect of 9-methoxyellipticine ($OCH_3$, upper curve) and of 9-hydroxyellipticine (−OH, lower curve). Results are expressed as a percentage of the fluorescence intensity, measured 6 min after additions, 100% corresponds to the intensity after addition of Triton X-100 (0.1% final concentration).

molar concentrations, but the protecting effect of the two dipolar ellipticines was weaker. This agrees with the differences noted in experiments with lipid monolayers [4, 5], and in quenching experiments, indicating that the more amphiphilic ellipticine derivatives were able to penetrate the lipid layers, while the dipolar drugs are involved with more superficial interactions. Penetration of the drug would bring about expansion of the membrane, and it has been proposed that such expansion is at the origin of the protection against the erythrocyte lysis observed at low anaesthetic concentrations [11, 12]. In agreement with this model, the amphiphilic 9-methoxyellipticine gave more protection against lysis, and at lower concentrations, than the dipolar 9-amino and 9-hydroxyellipticine.

It is also interesting to note that $2 \times 10^{-5}$ M 9-methoxyellipticine led to maximum leakage with liposomes (Fig. 4) and with resealed ghosts (Fig. 5). At this concentration, haemolysis began to take place in iso-osmotic conditions (Fig. 6) and there was complete lysis of erythrocytes placed in conditions corresponding to 20–30% haemolysis without drug. Moreover, it must be noted that with this concentration of 9-methoxyellipticine ($2 \times 10^{-5}$ M), the fluorescence of diphenylhexatriene inserted in erythrocyte membrane reached its minimum polarization rate (Fig. 3), indicating a maximum disordering effect of the drug.

This value does not correspond to the critical micellar concentration (C.M.C.) of the drug. The C.M.C was measured by determining the lowest drug

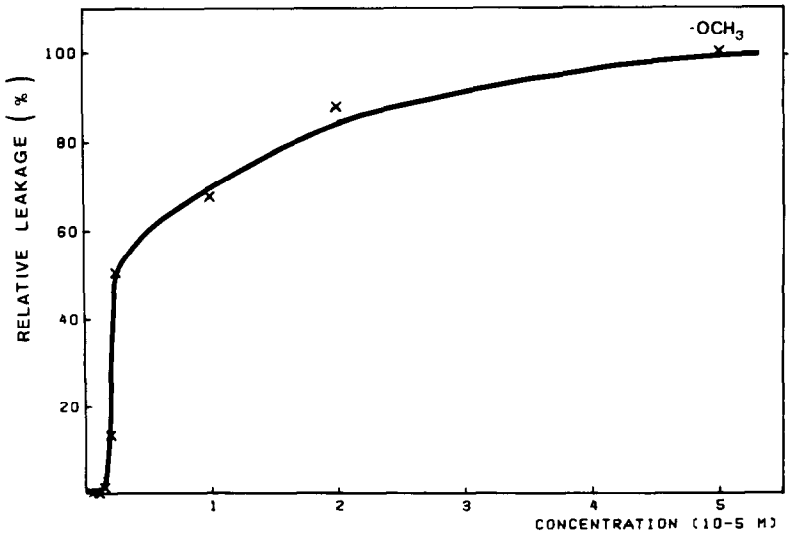


Fig. 5. Leakage of 6-carboxyfluoresceine encapsulated in resealed human erythrocytes: effect of 9-methoxyellipticine. Same conditions as in Fig. 4.

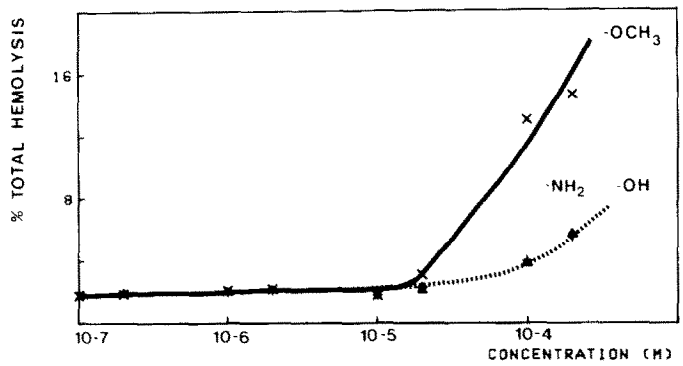


Fig. 6. Lysis of human erythrocytes in iso-osmotic medium in the presence of ellipticine derivatives. —$OCH_3$: 9-methoxyellipticine; —$NH_2$: 9-aminoellipticine; —OH: 9-hydroxyellipticine. Results are expressed as a percentage (100% corresponds to 20 min incubation in water).

concentration that gave a detectable reduction of water-surface tension, i.e. corresponding to the formation of a film at the air–water interface. For the three drugs tested the C.M.C. was between $2 \times 10^{-4}$ and $5 \times 10^{-4}$ M. The three drugs thus had lytic effects below their c.m.c. values.

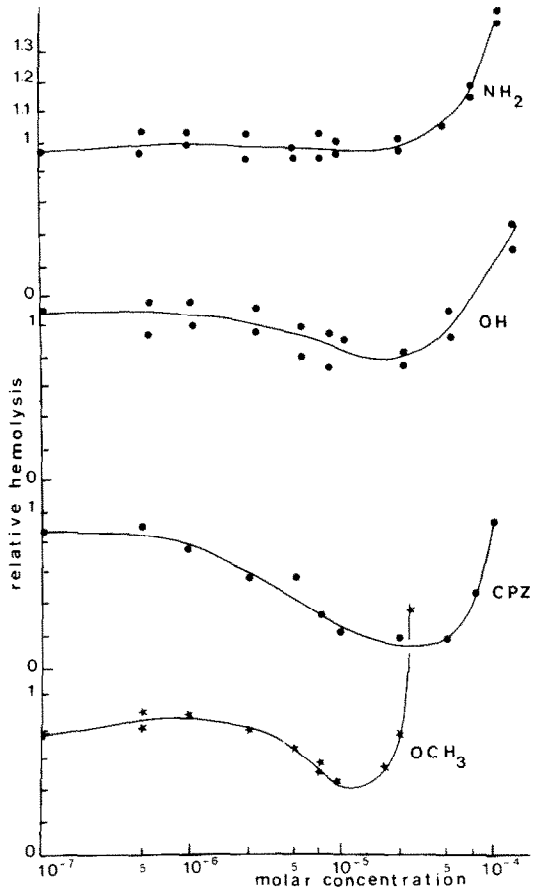


Fig. 7. Antihaemolytic effects of ellipticine derivatives. $OCH_3$: 9-methoxyellipticine; OH: 9-hydroxyellipticine; $NH_2$: 9-aminoellipticine; CPZ: chlorpromazine. Results are expressed as the ratio of the assay absorbance to the reference absorbance (the reference presented 20–30% of the total haemolysis). Values below 1 correspond to a protection against haemolysis, values above 1 to an increased haemolysis.

*Inhibition of respiration*

The inhibitory effect of the ellipticine derivatives was tested on the membrane isolated from *M. luteus*, the phospholipids of which are all anionic [6, 8].

The three drugs were active without delay and gave similar curves on plotting the inhibition percentage vs the drug concentration (Fig. 8). It must be noted that a similar efficiency of the three drugs was also obtained in the experiments on the drug effect on the electric charge of liposomes (Fig. 2).

*Conclusion*

The present paper completes and extends our previous findings on the effects of the ellipticine family anticancer drugs. These drugs present ionic interactions with membranes, and the more amphiphilic ones add hydrophobic interactions. The latter type of substances are able to penetrate natural membranes at least down to the carbonyl region of the phospholipids. These interactions lower the superficial charge of the membranes, bringing about disorganization of the structure, which is even more pronounced with drugs that can have hydrophobic interactions with the lipid phase. This disorganization will clearly disturb membrane functions.

The affinity of these drugs for membranes can explain observations that have been made during tests on animals. For instance, the very fast disappearance of the drug administered intravenously (90% eliminated within 2 min) [1]: this must be due to the drug binding onto blood cells. This binding can result in erythrocyte lysis, as observed when injections are given too fast. The neurological side effects could also be due to the affinity of ellipticines for membranes, since they seem to be at least as efficient against membrane systems as chlorpromazine.

The effects on membranes have to be considered in order to understand not only the toxic effects of ellipticines, but also their pharmacological fate. Their distribution in the organism, or within cells, is certainly influenced by their affinity for membranes, and one can also imagine that their cytotoxic

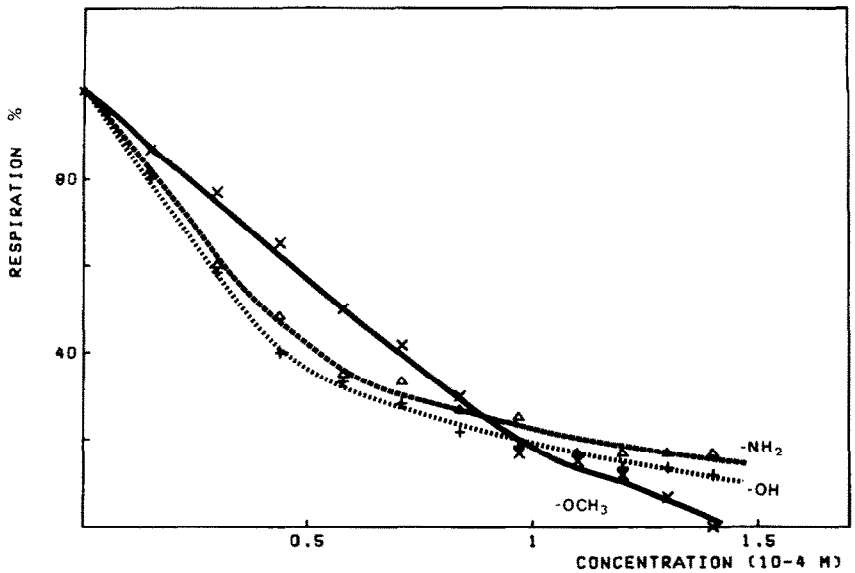


Fig. 8. Respiration inhibition of *M. luteus* membrane by ellipticine derivatives. —$OCH_3$: 9-methoxyellipticine; —OH: 9-hydroxyellipticine; —$NH_2$: 9-aminoellipticine. Substrate: L-malate. Drugs were added to the measurement cell. 100% corresponds to the oxygen consumed in the absence of drug.

effects, thus their antitumoural activity, are influenced by their ability to modify membrane properties. Depending on the balance between hydrophobic and ionic interactions between membranes and drugs, the above-mentioned effects could occur to different extents and have different consequences on membrane properties. Thus it would be useful to consider ellipticine–membrane interactions in the development of less toxic and/or more active ellipticine derivatives.

*Acknowledgements*—We thank Pr. R. Bierme (Hématologie—Toulouse, Rangueil) for the generous gift of human erythrocytes. This work was supported by a P.I.R.M.E.D. grant (A.S.P. antitumoraux).

## REFERENCES

1. K. W. Kohn, W. E. Ross and G. Glaubiger, in *Antibiotics* Vol. 5, Part 2, p. 195. Springer, New York (1979).
2. C. Paoletti, J. B. Le Pecq, N. Dat-Xuong, P. Juret, H. Garnier, J. L. Amiel and J. Rouesse, *Recent Results Canc. Res.* **74**, 107 (1980).
3. J. B. Le Pecq, N. Dat-Xuong, C. Gosse and C. Paoletti, *Proc. natn. Acad. Sci. U.S.A.* **71**, 5078 (1974).
4. E. M. El Mashak, C. Paoletti and J. F. Tocanne, *FEBS Lett.* **107**, 155 (1979).
5. E. M. El Mashak and J. F. Tocanne, *Eur. J. Biochem.* **105**, 193 (1980).
6. F. Tercé, J. F. Tocanne and G. Lanéelle, *Eur. J. Biochem.* **125**, 203 (1982).
7. M. B. Lees, in *Methods in Enzymology* Vol, III, p. 331. Academic Press, New York (1957).
8. F. Tercé, M. Gillois and G. Lanéelle, *FEMS microbiol. Lett.* **6**, 357 (1979).
9. S. Lepke and H. Passow, *Biochim. biophys. Acta* **255**, 696 (1972).
10. H. Machleidt, S. Roth and P. Seeman, *Biochim. biophys. Acta* **255**, 178 (1972).
11. P. Seeman, *Pharmac. Rev.* **24**, 583 (1972).
12. J. R. Trudell, *Biochim. biophys. Acta* **470**, 509 (1977).