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Enhancement of Cellular Uptake of Ellipticine by Insulin Preincubation*

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Abstract—*Ellipticine is a uleine alkaloid whose cytotxicity is a function of the ability of its cationic form to intercalate with helical nucleic acids. This study explores the uptake and binding of this antitumor alkaloid by cells of human breast cancer MDA-MB-231, sarcoma 180, several human lymphoblasts and one non-Hodgkin's lymphoma. In cells responsive to metabolic stimulation by insulin (MDA-MB-231; lymphoblasts), preincubation with this hormone enhanced cellular uptake of ellipticine, possibly by a pH loading mechanism dependent on increased metabolic activity and lowered intracellular pH. As a result, the amount of ellipticine associated with DNA increased, and there was increased cytotoxicity. This effect was not seen in sarcoma 180 or the non-Hodgkin's lymphoma samples.*

INTRODUCTION

EXTRACTS of the related plant species, *Ochrosia moorei* and *Excavatia coccinea*, were originally found to be active against sarcoma 180, adenocarcinoma 755 and L1210 leukemia *in vivo*, and to be cytotoxic for KB cells (human nasopharyngeal carcinoma) in tissue culture [1, 2]. The anti-tumor activity was subsequently found to be associated with the uleine alkaloids, ellipticine and 9-methoxyellipticine [3]. The pharmacology of these alkaloids has been reviewed [4]. Inhibition of cellular growth by ellipticine is dose-related in L1210 cells and cannot be eliminated by removal of the drug after a contact period of 15–20 min [5]. Ellipticine inhibits the processing of 45S nucleolar RNA into other species in the sequence of ribosomal RNA maturation in L1210 cells [6]. In a study of the ability of synchronized Chinese hamster cells to traverse the cell cycle, it was found that ellipticine inhibited the initiation of DNA synthesis and the completion of the G_2 phase, but was ineffective in preventing DNA synthesis in cells that were already in S phase at the time of exposure to drug [7]. Bhuyan *et al.* [8] reported that ellipticine inhibited DNA and RNA synthesis to a greater extent than protein synthesis in both Chinese hamster and L1210 cell lines. Alkaloids of this group are also known to induce breakage of DNA [9] and chromosomal aberrations [8]. All this evidence is compatible with the finding that drugs of this class are intercalating agents for nucleic acids [10, 11].

Ellipticine (pK_a 5.8) at pH 7.4 is mostly in the uncharged form, which is more lipid-soluble and better able to cross membranes; it becomes protonated under mild acidic conditions. The build-up of metabolic acids in a rapidly metabolizing cell should lower the pH of the intracellular milieu and cause more ellipticine to convert to its cationic form, which cannot cross the cell membrane and diffuse out of the cell. More uncharged drug should then diffuse into the cell to re-establish equilibrium and, in the absence of an active transport mechanism, ellipticine might achieve intracellular concentrations higher than those in the plasma. It has been observed that only the cationic form of ellipticine binds to helical DNA, and to helical forms of RNA such as transfer RNA [12]. Thus, by lowering intracellular pH, one should be able to increase both total intracellular ellipticine and the degree of intercalative interaction with nucleic acids. This cellular pharmacokinetic approach to enhancement of antitumor efficacy is explored in this paper.

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MATERIALS AND METHODS

Chemicals and tissues

Ellipticine (NSC-71795) was provided by Drs. David Abraham and Harry Wood, Drug Development Branch of the National Cancer Institute, Bethesda, MD. Sarcoma 180 (S180) in the ascites form was maintained in Swiss white mice (CD-1; Charles River Breeding Labs., N. Wilmington, MA) and harvested 4–6 days after i.p. inoculation of 10^6 cells. Human breast carcinoma MDA-MB-231 cells (gift of Dr. Carolyn Damsky, Wistar Institute, Philadelphia, PA) were maintained in Minimal Essential Medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, BME Vitamins (Flow Laboratories), 10% tryptose phosphate broth, 1000 units/ml penicillin–streptomycin (Grand Island Biological Co.) and 10 μg/ml insulin (bovine pancreas; Sigma Chemical Co.), which was sterilized by passage through a Falcon 0.22 micron filter. The MDA-MB-231 cells were passaged in a 1:5 weekly splitting, which resulted in the addition of approximately 10^6 viable cells to each 74 cm^2 flask. Thymidine-methyl-^{3}[H] (6.7 Ci/m mole) was purchased from the New England Nuclear Corp., deoxycholate from Fisher Scientific and 8-hydroxy-1,3,6-pyranetrisulfonic acid (pyranine) from Eastman Organics.

Ellipticine assay

Ellipticine content was measured in tissue culture cells harvested from flasks by scraping thoroughly with a rubber policeman and adding the cells suspended in medium to 15 ml centrifuge tubes. The cells were centrifuged and the pellet washed twice with 5 ml of saline; two further washes produced no more than a 10 per cent reduction in intracellular ellipticine. The minimum time for processing the zero time sample was 1 min, with exposure to the drug before centrifugation of less than 20 sec. One of two tubes of control cells was suspended in saline (5 ml), a drop of 0.4% trypan blue solution added and the viable cells counted in a hemocytometer. While there have been criticisms of the trypan blue exclusion technique, in our hands the correlation with subsequent cell growth was good. Generally, a 75 cm^2 flask of 3 days post 1:5 split cultures contained 3×10^6 viable cells. Three ml of extraction solution (0.5 M HCl in 50% ethanol) was added to each washed cell pellet and the cells were suspended and lysed. After centrifuging at 1000 ***g***, the supernatant was decanted into quartz tubes and ellipticine fluorescence was measured in a Turner fluorometer with filters 47B (for excitation) and 15 (for emission) . The standard curve of fluorescence vs ellipticine content was linear up to at least 400 ng per sample. There was no difference in the standard curves run with extracts from control vs insulin-treated cells. All fluorescence measurements were converted to 30 scale readings by the following factors: 4.48 for a 10–30 scale; 11.51 for a 3–30 scale; and 36.49 for a 1–30 scale.

Sample incubations

MDA-MB-231 cells were prepared for each experiment by aspirating the growth medium and adding 3 ml of phosphate-buffered saline (PBS) to wash the cells. PBS was then aspirated and 5 ml of complete medium without insulin supplement was added to each flask, which was then returned to the incubator for 1 hr. After this period, insulin at various concentrations was added to each flask, which was then re-incubated for the times indicated in the Results Section. Blast cells were removed from human blood samples with a Ficoll–Hypaque solution (Pharmacia), the cells washed twice and suspended in 8 ml of Hanks' balanced salt solution. Eight 1 ml samples of cell suspensions in Hanks' solution were incubated for 1 hr and one sample was checked for viable cells with trypan blue. Five of the remaining 7 samples containing various concentrations of insulin were incubated for 3 hr at 37°C. Ellipticine (0.1 μg) was added to 6 of the 7 flasks, which were incubated for 15 min and harvested as described above.

Ellipticine in DNA

Cell culture flasks were incubated from day 1 to day 3 after 1:5 split with 0.01 μ Ci of thymidine-^{3}[H] to label DNA for identification after fractionation. Once the location of the DNA band was established, MDA-MB-231 cells were no longer incubated with radioactive thymidine, but u.v. absorbance at 254 nm was used alone. Logarithmically-growing cells (3 days post 1:5 split) were incubated as described above, but the pellet of washed cells was suspended in 1 ml of a 5% *p*-aminosalicyclic acid–1% deoxycholate solution to lyse the cells. Sucrose density gradients were prepared with 1 M NaCl and 0.01 M EDTA as the light solution, and 1 M NaCl, 0.01 M EDTA and 31% (w/v) sucrose as the heavy solution, in a Beckamn Density Gradient Former set for a range of 30–90 to give a 5–20% gradient. One ml of cells in lysing solution was layered atop each gradient in cellulose nitrate tubes and the tubes were spun for 16 hr at 15,000 ***g*** in an SW-36 swinging bucket rotor using a Beckman L5-50 ultracentrifuge. The tubes were punctured at

the bottom and fractionated in an Isco model 640 Density Gradient Fractionator by passing a 35% sucrose solution up through the tube and collecting 1.2 ml fractions. As seen in Fig. 1, fractions 7, 8 and 9 contained the entire DNA band, as measured by both radioactivity (Packard Tri-Carb liquid scintillation spectrometer) and absorbance at 254 nm (Isco UA-2 ultraviolet analyzer). These fractions were pooled for ellipticine assay.

RESULTS

Cellular uptake of ellipticine

Figure 2 illustrates the time course of ellipticine uptake *in vitro* by logarithmically-growing MDA-MB-231 cells during 1 hr at drug concentrations of 0.01 and 0.1 μg/ml. Maximal uptake was reached 15–25 min after addition of drug; there was a slow fall during the following 35 min. Thus, all subsequent incubations with ellipticine were carried out for 15 min. The

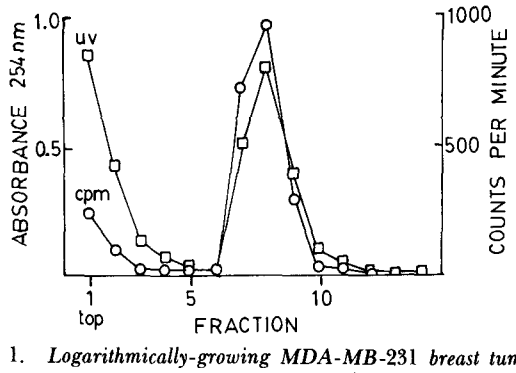

Fig. 1. Logarithmically-growing MDA-MB-231 breast tumor cells were grown in the presence of thymidine-[^{3}H], lysed and layered atop a 5–20% sucrose gradient, as described in the Materials and Methods section. Fractions (1.2 ml) were monitored by u.v. absorbance at 254 nm and each fraction was counted for tritium to locate the DNA band.

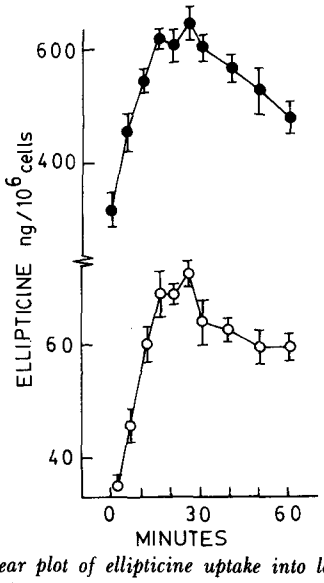

Fig. 2. Linear plot of ellipticine uptake into logarithmically-growing (MDA-MB-231 cells) as a function of time for 0.1 μg/ml (●—●) and 0.01 μg/ml (○—○) ellipticine. Initial values were obtained at 1 min.

growth rate of MDA-MB-231 cells can be regulated by the amount of supplemental insulin in the medium. Logarithmically-growing human breast tumor cells were incubated with ellipticine at 0.01 μg/ml for 15 min. In Fig. 3 it can be seen that 3 hr of preincubation with insulin gives maximum ellipticine uptake at both levels of insulin examined. There was a rapid increase in ellipticine uptake during the first hr and a slow but steady decline in intracellular ellipticine after more than 3 hr of high insulin concentrations. In the absence of insulin, ellipticine uptake occurred for only about 15 min.

Uptake of ellipticine, present at 6 different concentrations, was studied as a function of the level of insulin present during preincubation. Data of the type shown in Fig. 4 indicate that

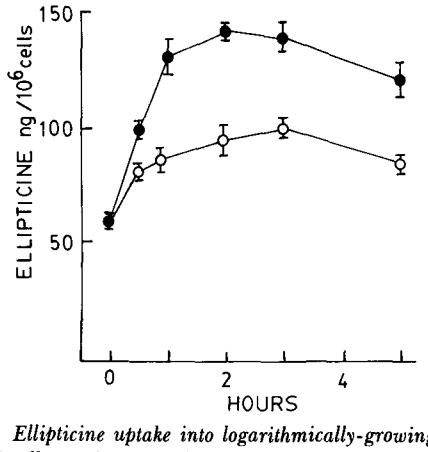

Fig. 3. Ellipticine uptake into logarithmically-growing MDA-MB-231 cells as a function of the time of insulin preincubation for 1 μg/ml (●—●) and 0.01 μg/ml (○—○) insulin; ellipticine at 0.01 μg/ml. Prolonged incubation of cells in insulin-free medium was not possible, due to deterioration of the cells; increase in intracellular drug only occurred during the first 15 min.

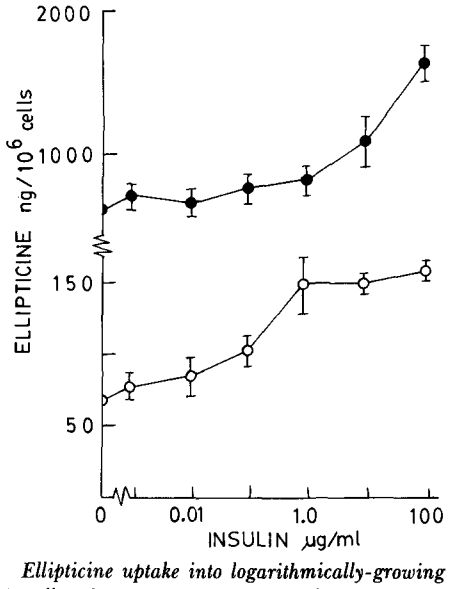

Fig. 4. Ellipticine uptake into logarithmically-growing MDA-MB-231 cells when incubated for 15 min with ellipticine at 0.01 μg/ml (○—○) and 0.1 μg/ml (●—●) as a function of the log of the insulin concentration during a 3 hr preincubation.

ellipticine uptake by log phase MDA-MB-231 cells increases with increasing insulin concentration. In contrast, sarcoma 180 cells, which do not exhibit growth dependence on insulin, and stationary phase MDA-MB-231 cells (confluent growth 6 days post 1:5 split) do not show significantly enhanced ellipticine uptake with increasing levels of insulin (Fig. 5). Statistically significant ($P < 0.05$) increase of ellipticine uptake occurred at lower insulin concentrations as the level of ellipticine was reduced (Fig. 6). This was only seen with log phase cells.

Ellipticine associated with DNA

Since only the cationic form of ellipticine is thought to be capable of binding DNA [12], cells which demonstrate enhanced ellipticine uptake with insulin preincubation should also have most of the intracellular ellipticine in the cationic form; thus, more ellipticine should be bound to DNA—its site of cytotoxic action. DNA fractions were analyzed for ellipticine to yield data illustrated in Fig. 7. At insulin concentrations of 0.1, 1.0 and 10.0 μg/ml, there was a statistically significant greater ellipticine fluorescence associated with DNA than for the control preparations. The data indicate that in MDA-MB-231 cells, insulin receptor stimulation leads to greater intracellular levels of ellipticine available for binding to DNA and execution of its cytotoxic effects. Dissociation of complex could complicate these data, but we found that dialysis of DNA-associated drug led to only very slow dissociation of the complex, with a maximum loss of about 20% in 24 hours.

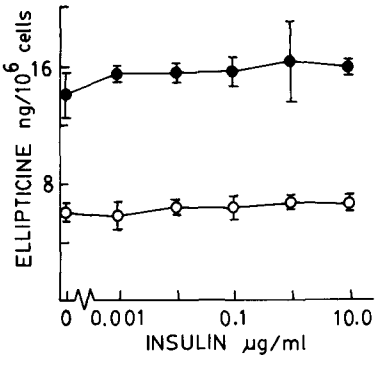

Fig. 5. Ellipticine uptake into stationary-growth DMA-MB-231 cells when incubated for 15 min at ellipticine concentrations of 0.05 μg/ml (●—●) and 0.02 μg/ml (○—○) as a function of the log of the insulin concentration during a 3 hr preincubation.

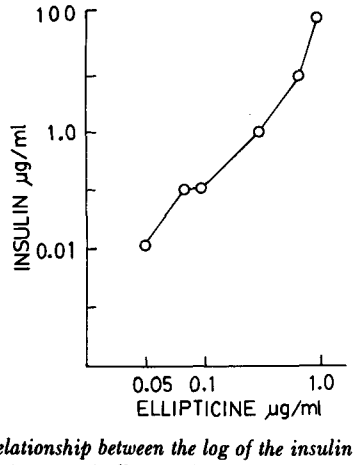

Fig. 6. Relationship between the log of the insulin concentration required to give statistically significant change in ellipticine uptake and the log of the ellipticine concentration for logarithmically-growing MDA-MB-231 cells. Dose–response curves of ellipticine uptake vs external ellipticine concentration were performed in quadruplicate at each level of insulin, and P values for the differences calculated, 0.05 being taken as the level of significance. The lowest ellipticine level was 0.05 μg/ml.

Ellipticine uptake in human tumor samples

Four human blood samples from newly-diagnosed patients were examined. Three of these untreated subjects had acute lymphoblastic leukemia (ALL) and the fourth had non-Hodgkin's lymphoma (NHL). These cells were incubated in the normal way with both insulin and 0.01 μg/ml ellipticine, and the amount of fluorescent drug within the cell was assayed. There was a statistically significant increase in ellipticine uptake for the ALL samples preincubated with insulin, but there was no appreciable change in drug uptake by NHL cells after preincubation with insulin at levels up to 10 μg/ml (Fig. 8).

Cytotoxicity studies

Cytotoxicity of ellipticine was determined by incubating MDA-MB-231 or S180 cells with various concentrations of ellipticine. It was apparent that in the breast cell cultures, the degree of inhibition obtained at 0.01 μg/ml of drug (approximately 85%) was achieved only by raising ellipticine levels ten-fold to 0.1 μg/ml in S180 cells (Fig. 9). Furthermore, supplementation of the medium with insulin up to 10 μg/ml had no effect on the cytotoxicity of

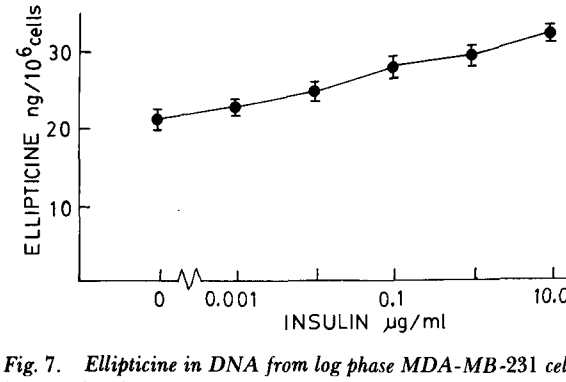

Fig. 7. Ellipticine in DNA from log phase MDA-MB-231 cells incubated with 0.1 μg/ml of ellipticine for 15 min as a function of the log of the insulin concentration present during a 3 hr period.

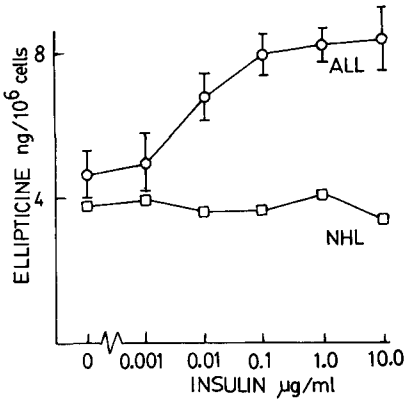

Fig. 8. Ellipticine uptake into human tumor samples as a function of the log of the insulin concentrations. (ALL, acute lymphoblastic leukemia; NHL, non-Hodgkin's lymphoma).

ellipticine, in keeping with uptake data. Since MDA-MB-231 cells do not grow, and indeed soon deteriorate, in the absence of insulin, attempts to show that insulin increased the cytotoxicity of ellipticine had to rely on short-term exposures. Cells were harvested in the normal fashion, allowed to grow for one day and then put into medium with or without insulin (10 μg/ml), but containing ellipticine (0.01 μg/ml), for 1.5 hr. At the end of this time the medium was changed to regular medium with insulin, but no ellipticine, and growth curves were determined. Cytotoxicity was evident in one day by a fall in the number of cells from 8×10^5 to $2.8 \pm 0.5 \times 10^5$ and $1.6 \pm 0.3 \times 10^5$ (5 values) for flasks containing ellipticine and ellipticine plus insulin respectively. In the absence of both insulin and drug, the cell number was 9×10^5.

DISCUSSION

The data presented in this paper illustrate one of the pharmacologic principles of combination cancer chemotherapy [13]. In this case we have one therapeutically active drug, ellipticine, whose uptake and interaction with its target site of action is potentiated by a second, non-cytotoxic agent, insulin. Insulin is postulated to stimulate intermediary metabolic pathways, causing an intracellular build-up of metabolic acids such as lactic and pyruvic acids and CO_2 (Fig. 10).

Kohn *et al.* [12] reported that ellipticine is lipid-soluble and can only cross a cellular membrane rapidly in its free base form. We have established (Fig. 2) that ellipticine uptake into MDA-MB-231 cells is a rapid process, maximum intracellular levels being reached after 15 min contact with the cells *in vitro*. Ellipticine, with a pK_a of 5.8 [14], is mostly uncharged at neutral pH and becomes protonated under mildly acidic conditions. With a favorable pH gradient across the cell membrane due to insulin-induced intracellular acidity, there will be a higher proportion of the cationic form than of the free base form of ellipticine within the cell. As the free base form of the alkaloid is in equilibrium across the cell membrane, more ellipticine will diffuse into the cell to re-establish the equilibrium. The lower intracellular pH will increase the concentration of cationic drug relative to the extracellular milieu. Since it has been shown [12] that only the cationic form of ellipticine can undergo intercalation, this should lead to increased amounts of DNA-bound drugs, as was indeed found to be the

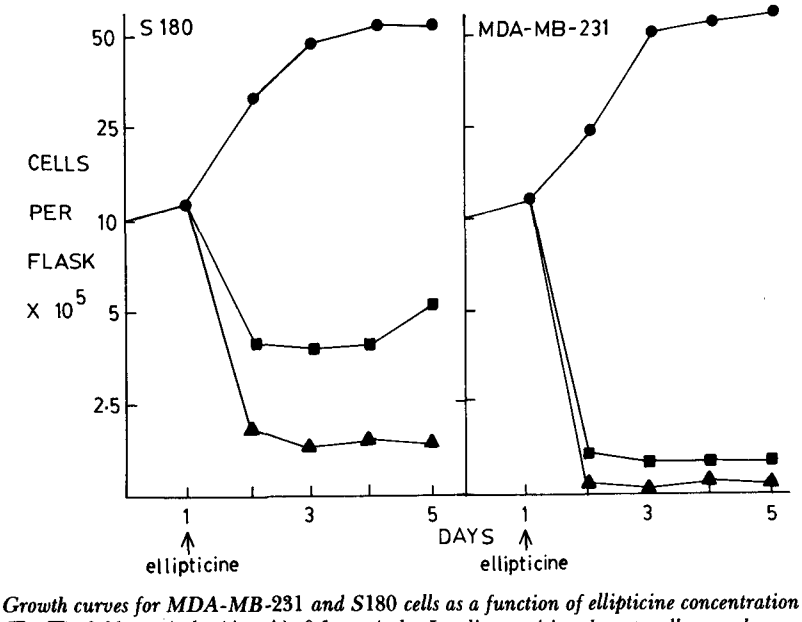

Fig. 9. Growth curves for MDA-MB-231 and S180 cells as a function of ellipticine concentration: (●—●) control; (■—■) 0.01 μg/ml; (▲—▲) 0.1 μg/ml. Insulin-sensitive breast cells are less resistant to ellipticine than S180 cells without insulin-dependence.

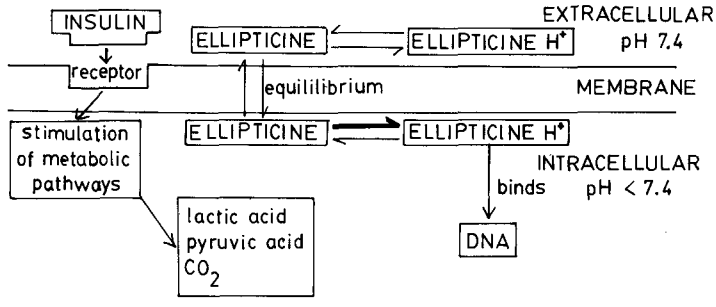

Fig. 10. Diagram of proposed mechanism of enhanced ellipticine uptake.

case in these studies (Fig. 7). While it may not appear to be a very marked effect, the nearly 50% increase in bound drug could create major distortion of DNA configuration. The overall schema is illustrated in Fig. 10. In contrast to log growth MDA-MB-231 cells, the same cells in stationary phase, and S180 cells, do not show dose-dependent stimulation of ellipticine uptake by insulin. S180 also shows no stimulation of its growth rate by insulin and thus probably lacks receptors for this hormone.

We have attempted to follow changes in intracellular pH by the use of pyranine, a pH probe that shows enhancement of fluorescent intensity as the pH falls. There was a marked increase in intracellular pyranine fluorescence as a result of preincubation with insulin (Fig. 11) only in cells sensitive to insulin, in this case those in exponential growth. Pyranine solutions show a 54% increase in fluorescence as the pH falls from 7 to 6. In Fig. 11 a similar increase is evident on going from an insulin level of 0.001 to one of 1.0 μg/ml, suggestive of a one pH unit drop intracellularly. The data, however, did not rule out the possibility that we were merely measuring enhanced uptake or retention of this material; this is the subject of continuing study.

While we have stressed here the likelihood of modification of intracellular pH being the mechanism for increased ellipticine uptake, it is not improbable that modulation of membrane properties through insulin interaction at the receptors will increase drug transport by itself. This aspect, as well as exploration of the possibility of practical chemotherapeutic applications, forms subjects for future study.

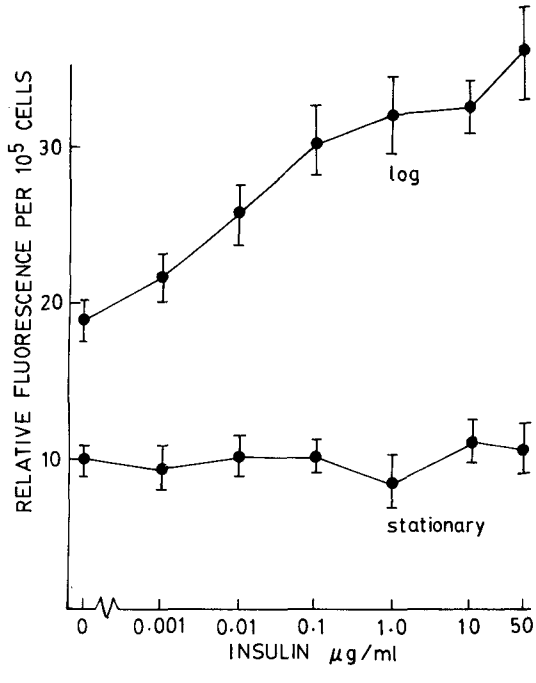

Fig. 11. Effect of insulin on the pyranine fluorescence of MDA-MB-231 cells incubated with hormone and dye for 1 hr *at* 37°C.

REFERENCES

1. Culvenor CCJ, Loder JW. Ellipticine alkaloids from *Ochrosia* and *Excavatia* species. Isolation and synthesis. 152*nd Meeting of the American Chemical Society, New York City* 1966; p. 29.
2. Dalton LK, Demerac S, Elmes BC, Loder JW, Swan JM, Teitei I. Synthesis of tumor inhibitory alkaloids, ellipticine, 9-methoxy-ellipticine, and related pyrido (4,3-b) carbazoles. *Aust J Chem* 1967; **20**: 2715–2727.
3. Svoboda GH, Poore GA, Montfort ML. Isolation of the alkaloids and study of the antitumor properties of 9-methoxyellipticine. *J Pharm Sci* 1968; **57**: 1720–1725.
4. Creasey WA. Plant alkaloids. In: Becker FF, ed. *Cancer—A Comprehensive Treatise.* New York: Plenum Press, 1977: Vol. 5, p.p. 379–425.
5. Li LH, Cowie CM. Biochemical effects of ellipticine on leukemia L1210 cells. *Biochim Biophys Acta* 1974; **353**: 375–384.

6. SNYDER AL, KANN HE, KOHN KW. Inhibition of the processing of ribosomal precursor RNA by intercalating agents. *J Mol Biol* 1971; **58**: 555–565.
7. TOBEY RA. A simple, rapid technique for determination of the effects of chemotherapeutic agents on mammalian cell cycle traverse. *Cancer Res* 1972; **32**: 309–316.
8. BHUYAN BK, FRASER TJ, LI LH. Cell cycle phase specificity and biochemical effects of ellipticine on mammalian cells. *Cancer Res* 1972; **32**: 2538–2544.
9. PAOLETTI C, LESCA C, CROS S, MAHY C, AUCLAIRS C. Ellipticine and derivatives induce breakage of L1210 cells DNA *in vitro*. *Biochem Pharmacol* 1979; **28**: 345–350.
10. SAUCIER JM, FESTY B, LEPECQ JB. The change of the torsion of the DNA helix caused by intercalation. II. Measurement of the relative change of torsion induced by various alkylating drugs. *Biochimie* 1971; **53**: 973–980.
11. FESTY B, POISSON J, PAOLETTI C. A new DNA intercalating drug: Methoxy-9-ellipticine. *FEBS Lett* 1971; **17**: 321–323.
12. KOHN KW, WARING MJ, GLAUBIGER D, FRIEDMAN CA. Intercalative binding of ellipticine to DNA. *Cancer Res.* 1975; **35**: 71–76.
13. CREASEY WA. Pharmcological considerations in combination chemotherapy. *Pharmacol Ther A* 1977; **1**, 307–325.
14. SORACE RA, SHEID B. Cytotoxic effects of the lysosomotropic agents DNA-ellipticine, DNA-daunorubicin, and DNA-ellipticine: daunorubicin on L1210 leukemia cells. *Cancer Treat Rep* 1979; **63**: 43–51.