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SURFACE ACTIVITY, CELLULAR UPTAKE AND CYTOTOXICITY OF TRICYCLIC PSYCHOACTIVE DRUGS *IN VITRO**

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Abstract—Isolated rat hepatocytes and Chang liver cell cultures were used to determine the relationship between magnitude of uptake by cells and cytotoxic effects of the tricyclic drugs amitriptyline (AT), imipramine (IM) and chlorpromazine (CPZ). Cell injury was evaluated by the extent of leakage of cytoplasmic and lysosomal enzymes from cells to surrounding medium and by cytopathic changes seen under surface scanning electron microscopy after drug exposure. The order of toxicity and of increasing amounts of drug uptake by cells was identical: CPZ > AT > IM. At equimolar concentrations in the medium, the uptake of CPZ by rat hepatocytes or Chang cells was 5- to 10-fold greater than that of AT or IM. Surface activity of drugs was determined to calculate their surface excess. The rank order of the surface excess of the drugs correlated with the rank order of their degree of uptake by the cells suggesting that surface active properties could play a role in differences in bioavailability and toxicity of these drugs to liver cell membranes.

Hepatic injury is a rare but possible consequence of administration of tricyclic antidepressants in man [1, 2]. Chlorpromazine may cause subclinical abnormalities of liver function tests in 50 per cent of recipients [3]. The pathophysiology of cholestasis associated with the clinical use of these drugs is poorly understood [4]. Previous work in this laboratory has demonstrated a positive correlation between the cytotoxic effect on liver cell cultures *in vitro* and the surface activity of phenothiazines [5] and erythromycin derivatives [6, 7].

The surface activity of drugs may be relevant to their lipid–water partition coefficient and/or their capacity to be adsorbed onto the cell membrane interface. This study was designed to test the hypothesis that the *in vitro* hepatotoxic effects of some chemically and therapeutically related surfactant compounds may correlate with their different surface activities and the concentrations of the drugs in the cells.

The results comparing cytotoxicity, cellular uptake and surface excess of chlorpromazine (CPZ), imipramine (IM) and amitriptyline (AT) are presented.

METHODS

[^{35}S]Chlorpromazine hydrochloride (18.8 mCi/m-mole) and methylene [^{14}C]imipramine hydrochloride (10.5 mCi/m-mole) were purchased from Amersham, England, and 5,10 (11) [^{14}C]amitriptyline hydrochloride (1.5 mCi/m-mole) was purchased from Merck, Sharp & Dohme Research Laboratories, Rahway, NJ. Their radiochemical purity was greater than 98 per cent. Crystalline drugs were obtained from the following sources: chlorpromazine hydrochloride (Smith, Kline & French Laboratories, Philadelphia, PA), imipramine hydrochloride (USV Pharmaceutical Corp., Philadelphia, PA), and amitriptyline hydrochloride (Merck, Sharp & Dohme). Drugs were dissolved in propylene glycol (PG) and then diluted with either Medium 199 for Chang cell cultures experiments, or with L-15 medium for isolated rat hepatocytes experiments. The total concentration of PG in control and drug-containing medium was 1%. Preliminary experiments showed that this concentration of PG had no detectable toxic effect on the cells.

Determination of octanol–water partition coefficients

To separatory funnels were added: 5 ml of the aqueous solution (HBSS), 20 μl of a 10^{-1} M stock of unlabeled drug and 5 ml of the organic solution (octanol). The funnels were shaken for 2 min. The solutions were allowed to separate for 10 min. Five 50-μl samples were taken from the organic phase, 0.5 ml of the aqueous phase was discarded through the stem (to avoid possible contamination) and 2–3 ml of the organic phase was collected. From this, five 50-μl samples were taken into scintillation counting vials. Ten ml Scintisol was added and the scintillation determined by standard techniques. The number of counts in the organic phase divided by the counts in the aqueous phase gave the number considered as the octanol–water partition coefficient.

Preparation of isolated rat hepatocytes

Isolated hepatic cells were prepared by liver perfusion of male Sprague–Dawley rats, 220–250 g, by the method of Berry and Friend [8] as modified by Seglen [9]. The percentage of viable cells was determined by Trypan blue exclusion and by O_2 utilization before and after the introduction of 1 mM succinate into the medium [10]. The number of rat liver cells was expressed as mg of soluble cell protein, measured by the method of Lowry *et al.* [11]. One million cells equaled 2.5 ± 0.2 mg of soluble cell protein.

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Tissue culture

Chang cell cultures originally derived from human liver and now an established line in the American Type Culture Collection (CCl.13) were purchased from Flow Laboratories (Rockville, MD). Cell viability was tested by Trypan blue exclusion and cells were counted in a Coulter counter apparatus by standard techniques.

Drug uptake experiments

Isolated rat hepatocytes. Control or drug-containing medium was added to culture tubes containing 2×10^6 cells. For "zero" incubation time the tubes were centrifuged promptly after adding the drugs to the cells. For the 5-, 10-, 20- and 40-min incubation times, the hepatocytes were incubated on a roller drum (8 rev/min) at 37°. The uptake was terminated by centrifuging the cells at 50 *g* for 1 min followed by washing once with 5 ml of 0.9% NaCl and centrifuging again. The cells were lysed in 15 ml Scintisol (New England Nuclear, Boston, MA) and the radioactivity counted in a Packard Tricarb scintillation spectrometer. The counts were converted to drug uptake from the specific activity of the labeled drug.

Chang cell cultures. Medium 199 containing radiolabelled drug with the desired final concentration was added to the tubes containing the monolayer with 1×10^6 cells. They were incubated at 37° for 0, 5, 10 and 20 min. We arbitrarily called "zero" the incubation time which resulted from adding the drug solution to the culture, and decanting the medium in a manner that allowed the drug solution to be in contact with the monolayer attached to the glass of the culture tube for approximately 1 sec. For longer incubations the tubes were placed horizontally and the monolayers were covered with the drug solution for the respective times. After incubation the cell monolayers were washed once with 10 ml of 0.9% NaCl and the cells were lysed by adding 15 ml Scintisol. Drug uptake by cells was calculated as described above.

Drug cytotoxicity experiments

Isolated rat hepatocytes. Medium containing the drug was mixed with medium containing 1×10^6 cells and incubated for 30–120 min at 37°. The cell suspensions were centrifuged for 1 min at 50 *g* and the supernatant fraction was assayed for the activities of lactate dehydrogenase (LDH*) [12] and β-glucuronidase (BG†) [13] leaked from cells into the surrounding medium. As described in previous work [6], experiments were performed to exclude possible drug effects on the activities of these enzymes other than those resulting from injury to cell membranes.

Chang cell cultures. The drug solutions were introduced into culture tubes containing a monolayer with 1×10^6 Chang liver cells. After the respective incubation times at 37°, the cytotoxicity was quantitated by measuring the activity of LDH and BC, as mentioned earlier.

Surface excess

Each drug was dissolved in the same balanced salt solution as contained in tissue culture media and the surface tension was measured using a Cahn electrobalance at 21° as described in previous work [14]. Surface excess of a given drug solution was calculated from the surface tension data according to the formula of Gibbs (cited by Adamson [15–17]) as follows: surface excess $(\Gamma) = 1/RT(\log \pi/\log C)\pi$; where $1/RT = 0.436 \times 10^{-10}$ mole/dyne · cm; C = concentration of the drug in moles; and π = surface pressure.

Surface scanning electron microscopy

Rat cells layered on coverslips treated with 1% poly–L–lysine were fixed in 2% isotonic gluteraldehyde in 0.2 M phosphate buffer, pH 7.4, for 24 hr at 4°. The coverslips were dehydrated through graded alcohol (30, 70, 95 and 100%, v/v), exposed to Freon 113, followed by critical point drying using Freon 13 in a critical point apparatus (OMAS SPC-1500). The slides were then coated with gold in a Technics Hummer Apparatus. Viewing and photography were done in a JEOL-JSM-35 scanning electron microscope using an accelerating voltage of 25 kV and a tilt of 0° or 45°.

Statistical analysis

The results were compared by analysis of variance, the Newman–Keuls test [18] and Dunnett's test [19]. A difference was considered to be significant when a $P < 0.01$ was obtained. Means ± S.E. are shown in the figures and the tables.

RESULTS

Isolated rat hepatocytes experiments

Drug uptake. Figure 1 shows the time course of

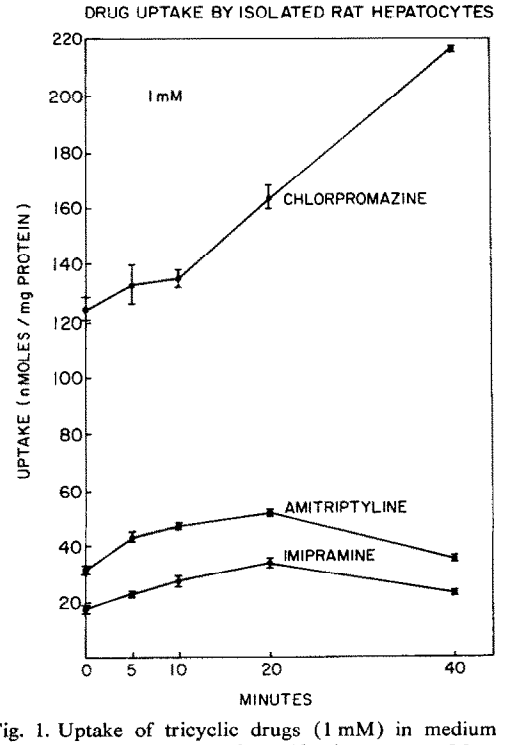

Fig. 1. Uptake of tricyclic drugs (1 mM) in medium by isolated rat hepatocytes at 0- to 40-min exposure. Means ± S.E. of four to six experiments are given. All three levels of uptake at any given time are statistically significantly different among themselves ($P < 0.01$).

*No. 1.1.1.27 (International Union of Biochemistry).
† No. 3.3.1.31 (International Union of Biochemistry).

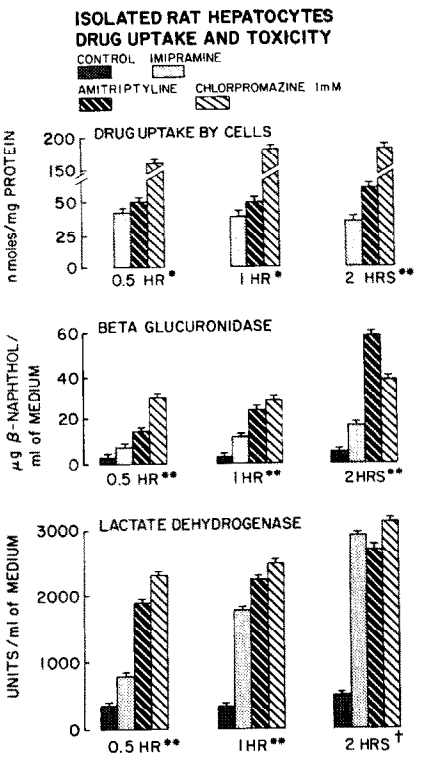

Fig. 2. Drug uptake of the tricyclic drugs and leakage of beta-glucuronidase and lactate dehydrogenase by isolated rat hepatocytes. Means ± S.E. of four to six experiments are given. Key: (*) chlorpromazine different from amitriptyline or imipramine ($P < 0.01$); (**) all different from each other ($P > 0.01$); and (†) all drugs different from control ($P < 0.01$).

uptake of drugs at a 1 mM concentration by hepatocytes. As much as 124, 31 and 17 nmoles/mg of protein of CPZ, AT and IM, respectively, were found to be with the cells at zero time. For AT and IM the uptake increased up to 20 min; there was some loss of drug from cells until experiment termination at 40 min. CPZ uptake was significantly greater than that of AT or IM and AT uptake was greater than that of IM.

Drug cytotoxicity. Enzyme leakage and drug uptake by rat hepatocytes exposed to a 1 mM concentration of drugs for different incubation times are shown in Fig. 2. The uptake of CPZ was the highest and that of IM was the lowest of all incubation times. Exposure to CPZ produced the greatest leakage of LDH and BG at 0.5 and 1 hr but at 2 hr AT produced the greatest leakage of BG.

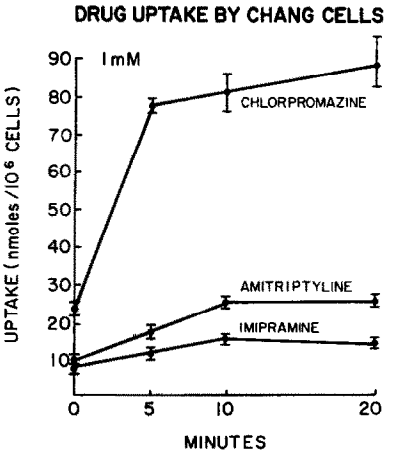

Fig. 3. Time course of the uptake of tricyclic drugs (1 mM) from medium by Chang cells in culture. Means ± S.E. of four to six experiments are given. All at any given time are statistically significantly different among themselves ($P < 0.01$) beginning at 5 min.

Chang cell cultures experiment

Drug uptake. Figure 3 shows the time course of the Chang cells' uptake of drugs at a 1 mM concentration in the medium. As much as 24, 9 and 8 nmoles/10^6 cells of CPZ, AT and IM, respectively, were found to be with the cells at zero time. Following that, CPZ uptake reached a plateau at 5 min and that of AT and IM at 10 min. The uptake of CPZ was greater than that of AT or IM at all of the incubation times. Also, AT uptake was higher than that of IM.

To investigate concentration-dependent drug uptake by the cells, three different concentrations of AT and IM (0.1, 0.5 and 1 mM) in medium were tested. The results are shown in Table 1. The higher the concentrations of AT and IM in medium, the greater the uptake by the cells.

Drug cytotoxicity. Using 0.5 and 1 mM drug concentrations, the cytotoxic effects of the drugs, measured by LDH and BG leakage, are shown in Fig. 4. They were statistically different among the three drugs tested, and concentration dependent. At all incubations and concentrations tested, exposure to CPZ resulted in the greatest toxicity. In turn, AT produced significantly greater toxicity than IM.

Surface scanning electron microscopy

Changes in the plasma membrane were evident after exposure to drugs at 1 mM in medium for 0.5 hr and

Table 1. Uptake of AT and IM by Chang cells

Drug concn (mM)	Uptake (nmoles drug/10^6 Chang cells) 0 min	2 min	5 min	10 min	20 min
Amitriptyline					
1	7.8 ± 0.1	12.3 ± 0.9	14.9 ± 0.9	21.5 ± 0.6	22.4 ± 1.7
0.5	4.2 ± 0.7	7.9 ± 0.2	8.9 ± 1.2	10.0 ± 0.1	10.3 ± 0.5
0.1	1.0 ± 0.1	2.2 ± 0.1	2.4 ± 0.1	2.7 ± 0.4	3.1 ± 0.3
Imipramine					
1	5.5 ± 0.5	9.2 ± 0.8	10.2 ± 0.2	12.9 ± 1.0	12.4 ± 1.2
0.5	3.0 ± 0.3	6.4 ± 0.2	8.5 ± 0.3	9.5 ± 0.6	9.5 ± 0.7
0.1	1.0 ± 0.2	1.2 ± 0.1	1.7 ± 0.2	2.0 ± 0.4	2.4 ± 0.2

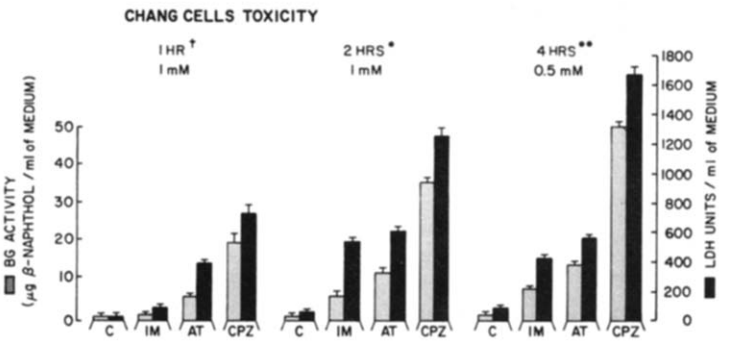

Fig. 4. Leakage of beta-glucuronidase (BG) and lactate dehydrogenase (LDH) from Chang cells in culture exposed to medium containing no drugs (C), imipramine (IM), amitriptyline (AT) or chlorpromazine (CPZ) at 1 mM for 1 or 2 hr or at 0.5 mM for 4 hr. Means ± S.E. of four to six experiments are given. LDH and BG are statistically significantly different ($P < 0.01$) among all drugs (**) except between IM and C for both enzymes (+) and IM and AT for LDH (*).

increased with greater concentrations. Hepatocytes exposed to IM were almost undifferentiable from controls (Fig. 5a); exposure to AT resulted in somewhat greater changes in the plasma membrane and slight deformity of the cells (Fig. 5b). The most marked alterations were evident after exposure to CPZ; the plasma membrane became grossly granulated and exhibited multiple bullae (Fig. 5c), some of which reached large sizes. At 2 hr there was bursting of some cells (Fig. 5d). These burst cells were not seen after incubation with the other drugs. The changes in Chang cells were of a similar nature and the differences among drugs were the same as with the rat liver cells.

Surface excess

The drugs under study are cationic surfactants. We examined the differences in this physicochemical property among the drugs and a possible relationship to differences in uptake and toxicity. Surface active drugs are known to accumulate at the interface in larger concentration than in the bulk of the medium (lipid–water interface in the case of the plasma membrane of cells in cultures or suspension); this is called surface excess and is related to, and calculated from the change of surface activity of the drugs with the change of their concentration using the formula of Gibbs described before.

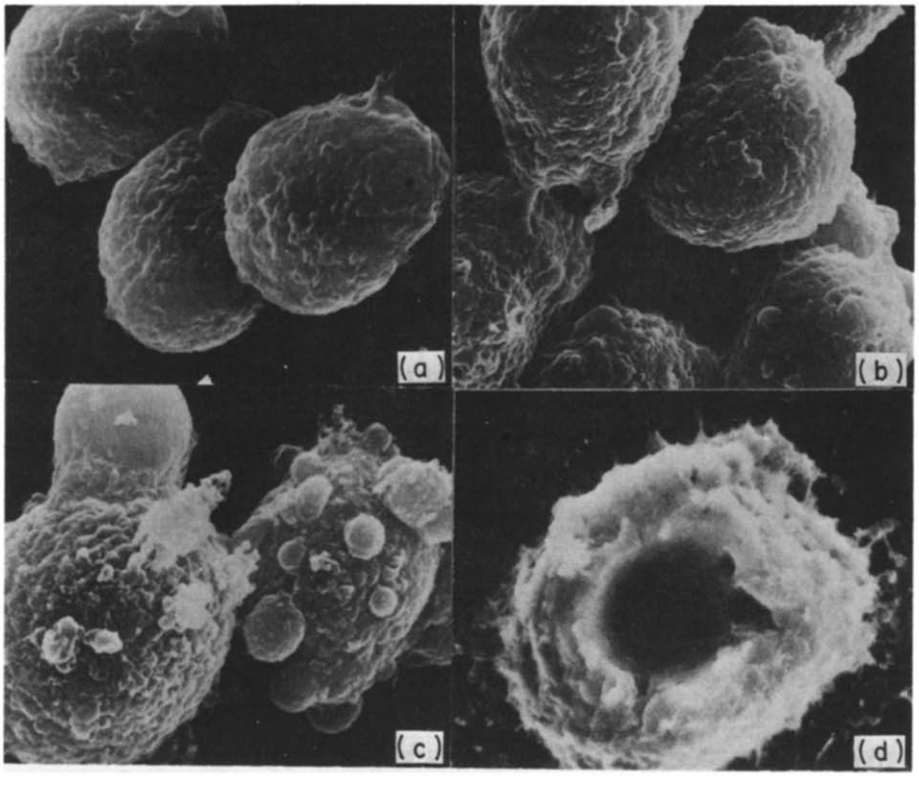

Fig. 5. Morphological appearance of cells under scanning electron microscopy after incubation for 0.5 hr in medium containing 1 mM imipramine (a) amitriptyline (b) or chlorpromazine (c). Chlorpromazine exposure for 2 hr is shown in panel d. Control cells are not shown because they are undifferentiable from those exposed to imipramine under these conditions.

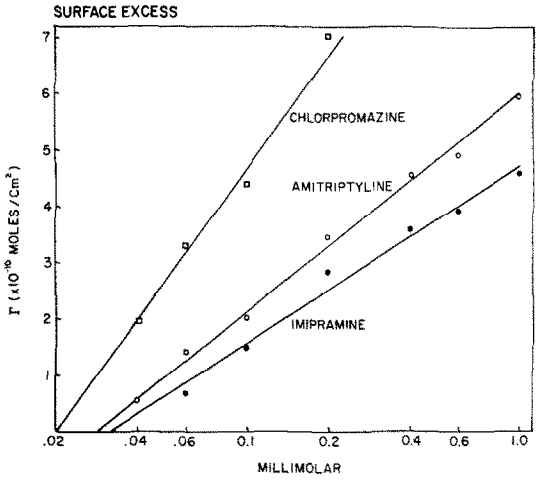

Fig. 6. Surface excess (Γ) of the tricyclic drugs determined by Gibbs equation (see Refs. 15–17). Γ (ordinate) is plotted against the logarithms of the drugs concentrations (abscissa).

A comparison of surface excess of solutions containing CPZ, AT and IM at various concentrations is shown in Fig. 6. The rank order of surface excess was CPZ > AT > IM; therefore, for any equal concentration in medium there would be a relatively greater accumulation of the respective drug at the interface for CPZ followed by AT and IM.

DISCUSSION

CPZ is considered the most effective psychoactive drug among the drugs tested in this experiment [20], is implicated most frequently in hepatotoxic occurrences among recipients [21] and has the most marked hepatotoxic effect *in vitro* [22, 23].

Our results indicate that CPZ was incorporated with the cells at levels four to ten times higher than those of AT or IM respectively. The dose- and time-dependent cytotoxicity *in vitro* and the extent of drug uptake by the cells followed the same order: CPZ > AT > IM. The similarity of results in both cell systems suggests that the differences in toxic effects among drugs results from the differences in their intrinsic physicochemical properties. We determined the octanol–water partitioning of the three drugs in question and also of promazine which is closely related chemically to chlorpromazine but has a markedly reduced surface activity. With the conditions of our *in vitro* experiments, the octanol–water partitioning ratios were practically identical for CPZ, IM and promazine (they were 46, 48 and 47, respectively) but the ratio was considerably lower for amitryptiline (which was 18). The results of the surface excess calculations shown in Fig. 6 show that the tricyclic drugs are potent surfactants, and their rank order of surface excess is identical to that of their cytotoxicity and uptake, but neither one parallels their octanol–water partitioning. The chemical structures of CPZ, AT and IM are shown in Fig. 7. The CPZ molecule is capable of interacting with the biological systems in at least two ways. First, the positively charged amine group on the side chain of the drug may electrostatically interact with the negatively charged carboxyl groups of the protein, glycoprotein and phospholipids of the cells [24–27]. Second, this ionic interaction is probably reinforced by hydrogen bonding between the sulfur and the chlorine of the drug molecule and the free hydroxyl groups of the carbohydrate residues of the glycoprotein or other macromolecules in the cell [26]. The lack of sulfur and chlorine atoms in the chemical structures of AT and IM causes a drastic drop in their surface activities, decreasing its adsorption properties and, consequently, their uptake and toxicity at the cells.

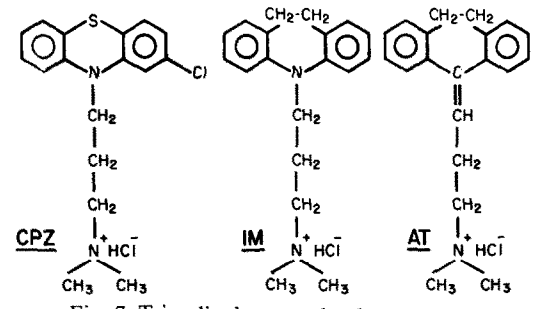

Fig. 7. Tricyclic drugs, molecular structure.

Our data suggest a correlation between the surface activity of a drug and the concentration of the drug achieved at an interface. The cell membrane interface concentration of the drug may then determine the extent of its therapeutic or toxic effects. It must be pointed out that the concentrations needed to demonstrate the differences among the drugs were much higher than those reported in the blood of patients treated with them; however, their concentrations in bile, liver parenchyma or liver cell receptors are unknown. Nevertheless, at the same high concentrations *in vitro* the effects were markedly different among these drugs; thus, the results are of value in the comparative sense.

Results of uptake and toxicity of surface active erythromycins [7] and these results with surface active psychoactive drugs demonstrate that the differences in *in vitro* hepatotoxicity among chemically related surfactant drugs correlate with their surface activities and their capacity to be incorporated within the cells. The final *in vivo* therapeutic and toxic implications of these *in vitro* findings remain to be elucidated.

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REFERENCES

1. J. Yon and S. Anuras, *J. Am. med. Ass.* **232**, 833 (1975).
2. M. D. Short, J. M. Burn and M. E. Harris, *J. Am. med. Ass.* **206**, 1791 (1968).
3. L. Waitzkin, *Ann. intern. Med.* **49**, 607 (1958).
4. G. L. Plaa and B. G. Priestly, *Pharmac. Rev.* **28**, 207 (1977).
5. C. A. Dujovne, G. Lavelle, P. Weiss, J. R. Bianchine and L. Lasagna, *Archs int. Pharmacodyn. Ther.* **186**, 84 (1970).
6. C. A. Dujovne, D. Shoeman, J. Bianchine and L. Lasagna, *J. Lab. clin. Med.* **79**, 832 (1972).

7. C. A. Dujovne, *Biochem. Pharmac.* **27**, 1925 (1978).
8. M. N. Berry and D. S. Friend, *J. Cell Biol.* **43**, 506 (1969).
9. P. O. Seglen, *Expl Cell Res.* **74**, 450 (1972).
10. H. Bauer, P. Kasperek and E. Pfaff, *Hoppe-Seyler's Z. physiol. Chem.* **356**, 827 (1975).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
12. F. Wroblewski and J. LaDue, *Proc. Soc. exp. Biol. Med.* **90**, 210 (1955).
13. L. J. Greenberg, *Analyt. Biochem.* **14**, 265 (1966).
14. C. A. Dujovne, *Toxic. appl. Pharmac.* **32**, 11 (1975).
15. A. W. Adamson, *Physical Chemistry of Surfaces*, 3rd Edn, p. 68. John Wiley, New York (1976).
16. A. W. Adamson, *Physical Chemistry of Surfaces*, 3rd Edn, p. 76. John Wiley, New York (1976).
17. A. W. Adamson, *Physical Chemistry of Surfaces*, 3rd Edn, p. 219. John Wiley, New York (1976).
18. B. J. Winer, *Statistical Principles in Experimental Design*, p. 191. McGraw-Hill, New York (1971).
19. C. W. Dunnett, *Biometrics* **20**, 482 (1964).
20. J. M. Davis and R. Casper, *Drugs* **14**, 260 (1977).
21. K. G. Ishak and N. S. Irey, *Archs Path.* **93**, 283 (1972).
22. C. A. Dujovne, R. Levy and J. H. Zimmerman, *Proc. Soc. exp. Biol. Med.* **128**, 561 (1969).
23. C. A. Dujovne and J. H. Zimmerman, *Proc. Soc. exp. Biol. Med.* **131**, 583 (1969).
24. D. N. Teller and H. C. B. Denber, *Dis. nerv. Syst.* **29**, 593 (1968).
25. A. E. Clarke and M. A. Denborough, *Clin. Chem.* **17**, 998 (1971).
26. A. E. Clarke, V. M. Martiz and M. A. Denborough, *Chem. Biol. Interact.* **5**, 265 (1972).
27. M. C. Carey, P. C. Hirom and D. M. Small, *Biochem. J.* **153**, 519 (1976).