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Chlorpromazine Uptake by Isolated Rat Hepatocytes (40534)[1]

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Introduction. Chlorpromazine (CPZ), a widely used psychotropic drug is known to have the capacity to produce biliary canalicular damage in man (1–3). The mechanism of such effect is unknown but the interactions of CPZ with bile proteins and liver cell membranes and its known surfactant properties have been suspected to play an important role in its therapeutic as well as toxic properties (4, 5). Although there is information on the secretion of CPZ in bile (6–8), little is known about its transport in the liver cells. This study is an investigation on the mechanisms involved in the uptake of CPZ by isolated rat liver cells.

Materials and methods. [^{35}S]Chlorpromazine chloride (specific activity 18.8 mCi/mole) was obtained from Radiochemical Centre (Amersham, England) with radiochemical purity greater than 98%. Crystalline chlorpromazine hydrochloride, oligomycin, carbonylcyanide *m*-chlorophenylhydrazone (CCP), and ouabain were obtained from New England Nuclear (Boston, Mass.). Collagenase was obtained from Worthington Biochemical Corporation (Freehold, N.J.). Silicon oil (d 1.05) was purchased from Aldrich Chemical (Milwaukee, Wis.). Hanks' balanced salt solution was purchased from Grand Island Biological Company (Grand Island, N.Y.). All other reagents used were commercially available products of analytical grade.

Hepatocytes were isolated form the livers of male Sprague–Dawley rats (220–250 g) which had free access to a standard pellet diet and water until they were anesthesized with ether prior to liver perfusion. Isolated hepatic cells were prepared by the method of Berry and Friend (9) as modified by Seglen (10); after collagenase perfusion, the isolated hepatocytes were suspended in Hanks' buffer solution at pH 7.2. Membrane integrity and viability of cells were determined by oxygen consumption before and after exposure to succinate as described by Baur *et al.* (11) and by Trypan blue exclusion; suspensions with 85–95% viable cells and a succinate ratio of less than 1.3 were considered suitable for the experiments.

The uptake studies required very short incubation periods, therefore, a rapid centrifugal filtration technique was used for the separation of cells from the medium (within 1–2 sec) as described by Schwarz *et al.* (12). Microtubes were prepared by layering from bottom to top: 50 μl of 3 *M* KOH, 100 μl of silicon oil, and 100 μl of Hanks' buffer solution containing various concentrations of labeled and unlabeled CPZ. The hepatocytes were preincubated at 37° for 5 min; the uptake experiment was started by introducing 100 μl of cell suspension containing approximately 10^5 cells to the top phase of the tube. The tubes were incubated for 5, 10, 20, 30, 45, and 60 sec. The uptake was terminated by separating the cells from the incubation medium by centrifugation at 10,000*g* for 10 sec. The pellet was collected by cutting the bottom end of the plastic tube just below the silicon layer and this cone was then placed in 0.4 ml protosol at 50° for 3 hr to dissolve the pellet. Scintisol was added, the radioactivity was counted in a Packard Scintillation spectrometer, and counts converted to nmole of drug per milligram of cell soluble protein. An aliquot of one million cells contained 2.48 ± 0.1 mg of cell soluble protein, determined by the method of Lowry *et al.* (13). The cellular uptake of drug was calculated after correcting for the residual fluid adhered to the pellet, the volume of which was calculated by adding [^{14}C]dextran to the incubating medium and determining the amount of [^{14}C]dextran with the pellet.

The effect on CPZ uptake by potassium cyanide, 2,4-dinitrophenol, ouabain, CCP,

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and oligomycin was determined by preincubating the cells for 5 min at 37° with one of the inhibitors. Then the cells were incubated directly with ^{35}S-labeled CPZ (100 μM). After incubation with shaking for 5 min, 200 μl of the cell suspension was transferred to the microtubes and centrifuged as described previosly for CPZ uptake. Since oligomycin was added as 0.4% ethanolic solution, preliminary control experiments were performed to evaluate the effects of ethanol. There was no significant difference between ethanol-treated and control cells under our experimental conditions. Cell viability was determined at the end of metabolic inhibitor experiments to rule out the possibility that changes in uptake resulted from toxicity rather than from metabolic inhibition.

To measure CPZ efflux, cell suspensions were incubated in a Dubnoff shaker, at 37° for 5 min, with radioactively labeled CPZ at a concentration of 10 μM. The cell suspensions were then centrifuged at 50g for 2 min, the supernatant was decanted, and the cells were resuspended in fresh Hanks' medium with or without 100 μM nonradioactively labeled CPZ and incubated for up to 20 sec (linear part of CPZ uptake). Aliquots of 100 μl of the supernatant were taken from each tube at 5-sec intervals and placed in scintillation vials; the radioactivity thus determined was utilized to calculate the rate of efflux of drug.

The data presented in this report are means and standard errors of the results in four different preparations for each experiment. Each experiment was repeated at least twice. The data were analyzed by Student's t test and linear regression analysis.

Results. The uptake of [^{35}S]CPZ at concentrations of 10 to 200 μM by rat hepatocytes at 37° is shown in Fig. 1. The use of higher concentrations in the medium was precluded because of erratic solubility of the drug and the occurrence of cytotoxicity, both of which interfered with accurate evaluation of the concentration of the drug in cells. After a very fast concentration-dependent uptake, influx was linear for up to 20 sec. From 30 to 60 sec, the uptake remained at a plateau, reflecting an equilibrium between influx and efflux of the drug.

The extrapolation of the linear part of the uptake curves to zero showed a positive intercept (Fig. 1). According to reports on previous investigations of this type done with bile acids, the positive intercept values represent a rapid adsorption of drug onto the cell membranes (nonspecific binding); the slope of the linear part of the uptake curve represents the initial uptake rate of absorption by the isolated hepatocytes (14, 15). The extent of adsorption as a function of CPZ concentration in medium is shown in Fig. 2A. The adsorption increased in linear relationship to the increase in drug concentration.

The initial rate of CPZ absorption is shown in Fig. 2B. The linearity of the initial rate of absorption at the drug concentrations tested would indicate that an unsaturable absorption process is involved and that the uptake of CPZ is passive and concentration dependent. The slope of the absorption curve represents the diffusion coefficient (14) which was calculated to be 0.15 nmole/mg protein/min/μM.

In comparing both the adsorption and the absorption at a specific concentration and time (10 μM, 5 sec), one can find an approximate adsorption to absorption ratio of 5 to 1, this ratio becoming smaller after the uptake reaches the equilibrium stage (ratio 1:1). Since the uptake rates were calculated after correcting for adsorption (Fig. 1), diffusion of CPZ may represent a process separate from adsorption.

The initial uptake of CPZ at various concentrations of cells in medium at 37° was determined after incubations of 5, 10, and 20 sec. The results are shown in Fig. 3. The

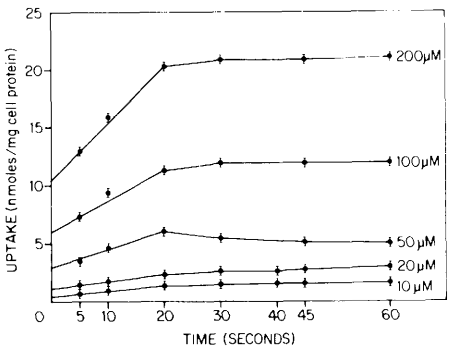

FIG. 1. Chlorpromazine uptake by rat hepatocytes incubated 5 to 60 sec with various concentrations of drug in medium. Mean and SE of four replicates.

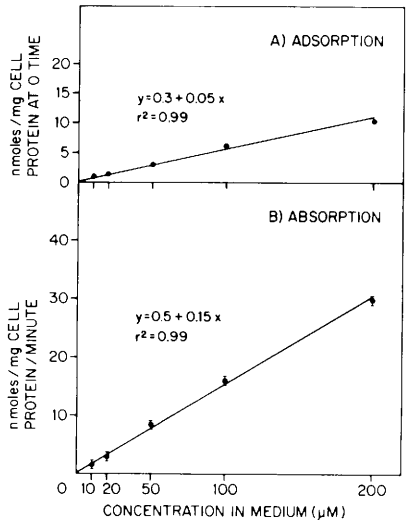

FIG. 2. Adsorption and absorption of chlorpromazine in relation to its concentration in medium. (A) Each point of the adsorption curve represents the zero-time intercept of the regression line for each of the five concentrations shown in Fig. 1. (B) Each point of the absorption curve represents the means and SE of the slope of the regression line through five data points at three different times (5, 10, and 20 sec) taken from Fig. 1. A regression line through these means is shown.

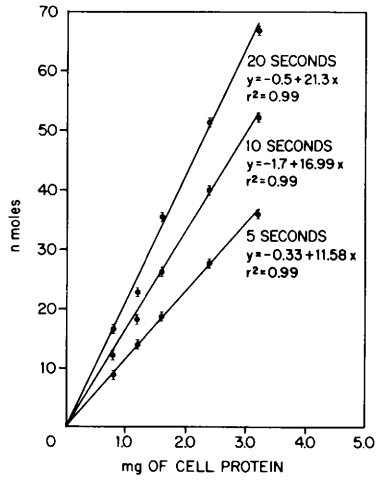

FIG. 3. Differences in uptake of chlorpromazine at 200 μM in medium at 5, 10, and 20 sec in relationship to the number of cells (mg of cell protein) exposed to the drug (mean and SE of four replicates).

uptake was linear at all concentrations used (0.8–3.2 mg protein/ml).

The effect of temperature on CPZ uptake was determined at 2 and 37°. At the lower temperature there was approximately a 40% decrease in the uptake of CPZ after 5 sec of incubation. There was no evidence that the solubility of CPZ at these concentrations was affected by the lower temperature.

The metabolic inhibitors tested had no significant effect on the uptake of CPZ after 5-min incubation, nor did they affect the viability of the cells (Table I).

The fluxes of CPZ in and out of isolated hepatocytes are shown in Fig. 4. There is a positive intercept of efflux curves at zero time. The extent but not the rate of efflux increased slightly after further addition of CPZ to the medium. This may be a phenomenon similar to that of "exchange diffusion" described for other drugs. The rate of influx was faster than that of efflux and the extent of influx was

TABLE I. EFFECTS OF METABOLIC INHIBITORS ON UPTAKE OF CHLORPROMAZINE (CPZ) BY ISOLATED RAT HEPATOCYTES[a]

Inhibitor concentration	CPZ (nmoles/mg protein)	Cell viability (%)
Control	9.13 ± 0.18[b]	83.9
Potassium cyanide (1 mM)	9.86 ± 0.26	84.7
2,4-Dinitrophenol (1 mM)	8.78 ± 0.25	83.8
Ouabain (1 mM)	9.65 ± 0.20	84.7
Carbonylcyanide *m*-chlorophenylhydrazone (1 mM)	8.78 ± 0.2	86.3
Oligomycin (20 μg/ml)	8.18 ± 0.4	83.7

[a] Cells (3 mg protein/ml) were incubated with different metabolic inhibitors for 5 min at 37°.
[b] Mean ± SE.

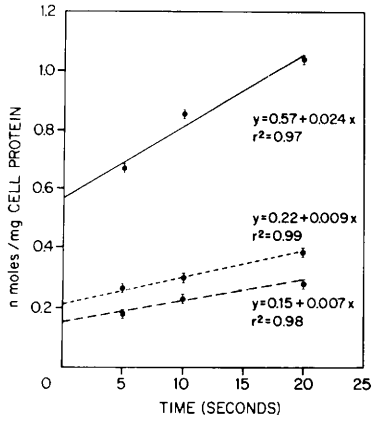

FIG. 4. Influx (solid line) and efflux of chlorpromazine in rat hepatocytes exposed to a 10 μM concentration of the drug for 5 min. Upper broken line represents efflux into medium with 100 μM chlorpromazine added; lower broken line represents efflux into medium without chlorpromazine added. Means and SE of four replicated experiments.

about threefold greater which indicates that a small portion of the drug already in the cell is available for the efflux processes. There was little difference in extent and none in the rate of efflux to medium with or without chlorpromazine suggesting that efflux is not related to the equilibrium between intra- and extracellular drug concentrations.

Discussion. Our results demonstrate that CPZ is rapidly and extensively adsorbed (nonspecific binding) onto isolated rat hepatocytes and that this phenomenon is drug concentration dependent. Two possible means of CPZ interaction with the cell plasma membranes could be suggested. The nonpolar part (a polycyclic system) may be adsorbed and intercalated into lipid membrane bilayers and bind to the phospholipid content of the cellular membrane. The polar part (a dimethylaminopropyl chain) which contains the positively charged amino group may cause an ionic interaction with the carboxyl groups on the membrane protein constituents, including glycoproteins. Such type of ionic interaction between the drug and cell proteins in human bile was proposed by Clarke *et al.* (16).

When the concentration of CPZ in the incubation solution is raised, the absorption rate is linearly related to the concentration. The lack of saturation kinetics observed is consistent with the absorption of CPZ through lipophilic areas of the membranes by passive diffusion. This uptake process could represent that the uptake is saturated only at very high concentrations. Imipramine which has chemical structural similarities with CPZ was also found by Bickel and Borner (17) to enter the rat liver from the perfusion medium by passive diffusion. In order to find out if the absorption process occurs against a concentration gradient ("concentrative"), the intracellular concentration of transported CPZ was calculated from the data shown in the results. At 20 sec of incubation with 10 μM CPZ, the ratio of intracellular to extracellular concentration was 33. A ratio of 32 was found for imipramine uptake by Bickel and Borner (17).

The lack of effect of metabolic inhibitors is consistent with the hypothesis that absorption of CPZ takes place by a passive diffusion process which is not energy dependent. CPZ is a strong cationic surfactant; the large extent of its adsorption onto the cell membranes offers ideal conditions for its accumulation onto canalicular membranes when the drug is secreted in bile *in vivo.* This should facilitate whatever effect CPZ may have on the structure and functions of the biliary pole of the hepatocellular membrane which may play a role in the capacity of CPZ to produce cholestasis in man (4–18).

Summary. Isolated rat hepatocytes were found to adsorb (nonspecific binding) as well as to absorb chlorpromazine. The linearity of chlorpromazine adsorption was dependent on the drug concentration in the incubation medium. Also, the absorption of chlorpromazine was linear with respect to the drug concentration, suggesting an unsaturable uptake process which was considered to represent simple diffusion, with a diffusion coefficient of 0.15 nmole chlorpromazine/mg protein/min/μM. Chlorpromazine uptake was not inhibited by the action of a series of metabolic inhibitors present in the reaction medium. The drug is rapidly and to a large extent adsorbed onto hepatocellular membranes.

1. Waitzkin, L., Ann. Intern. Med. **49,** 607 (1958).
2. Dickes, R., Schenker, V., and Deutsch, L., N. Engl. J. Med. **256,** 1 (1957).
3. Hollister, L. E., Amer. J. Med. **23,** 870 (1957).
4. Carey, M. C., Hirom, P. C., and Small, D. M., Biochem. J. **153,** 510 (1976).
5. Seeman, P. M., and Bialy, H. S., Biochem. Pharmacol. **12,** 1181 (1963).
6. Flanagan, T. L., Lin, T. H., Novick, W. J., Rondish, I. N., Bocker, C. A., and Van Loon, E. J., J. Med. Pharm. Chem. **1,** 263 (1959).
7. Flanagan, T. L., Reynolds, L. W., Novick, W. J., Lin, T. H., Rondish, I. M., and Van Loon, E. J., J. Pharm. Sci. **51,** 833 (1962).
8. Hanson, E., and Schmiterlow, C. G., Arch. Int. Pharmacodyn. **131,** 309 (1961).
9. Berry, M. N., and Friend, D. S., J. Cell Biol. **4,** 506 (1969).
10. Seglen, P. O., Exp. Cell Res. **74,** 450 (1975).
11. Bauer, H., Kasperek, S., and Pfaff, F., Hoppe-Seyler's Z. Physiol. Chem. **356,** 827 (1975).
12. Schwarz, L. R., Burr, R., Schwenk, M., Pfaff, E., and Greim, H., Eur. J. Biochem. **55,** 617 (1975).
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193,** 265 (1951).
14. Anwer, M. S., Kroker, R., and Hegner, D., Hoppe-Seyler's Z. Physiol. Chem. **357,** 1477 (1976).

15. Anwer, M. S., Kroker, R. S., and Hegner, D., Biochem. Biophys. Res. Commun. **73**, 63 (1976).
16. Clarke, A. E., Maritz, V. M., and Denborough, M. A., Chem. Biol. Interact. **5**, 265 (1972).
17. Bickel, M. H., and Borner, H., Naunyn-Schmiedeberg's Arch. Pharmacol. **284**, 339 (1974).
18. Salhab, A. S., Yasuhara, H., and Dujovne, C. A., Biochem. Pharmacol., in press.

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