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REFERENCES

1. E. C. C. LIM and W. E. KNOX, *Biochim. biophys. Acta* **26,** 85 (1957).
2. F. T. KENNEY and R. M. FLORA, *J. biol. Chem.* **236,** 2699 (1961).
3. F. T. KENNEY, *J. biol. Chem.* **237,** 1610 (1962).
4. E. REICH and J. H. GOLDBERG, *Prog. Nucleic Acid Res.* **3,** 183 (1964).
5. R. P. PERRY, *Expl Cell Res.* **29,** 400 (1963).
6. S. H. WILSON and M. B. HOAGLAND, *Biochem. J.* **103,** 557 (1967).
7. N. E. SLADEK and H. C. PITOT, *Cancer Res.* **30,** 1598 (1970).
8. A. S. A. DA SILVA, Doctorate Thesis, School of Medicine, Universidade de São Paulo (1971).
9. L. J. RATHER, *Bull. Johns Hopkins Hosp.* **88,** 38 (1951).
10. L. J. R: G. KLEINFELD, *Cancer Res.* **17,** 954 (1957).
11. H. R. ADAMS and H. BUSCH, *Cancer Res.* **23,** 576 (1963).
12. J. G. VILLALOBOS, JR., W. J. STEELE, JR. and H. BUSCH, *Biochim. biophys. Acta* **91,** 233 (1964).
13. J. G. VILLALOBOS, JR., W. J. STEELE, JR. and H. BUSCH, *Biochem. biophys. Res. Commun.* **17,** 723 (1964).
14. T. I. DIAMONDSTONE, *Analyt. Biochem.* **16,** 395 (1966).
15. S. GIEGEL, *Non-parametric Statistics*, p. 116. McGraw-Hill, New York (1956).
16. M. BRENTANI, M. KUBOTA and R. BRENTANI, *Biochem. J.* **130,** 11 (1972).
17. R. P. PERRY, J. LA TORRE, D. F. KELLY and J. R. GRENBERG, *J. Cell Physiol.* **72,** 235 (1968).
18. E. RANDERATH and K. RANDERATH, *Analyt. Biochem.* **12,** 83 (1965).
19. G. P. GEORGIEV and V. L. MANTIEVA, *Biochim. biophys. Acta* **61,** 153 (1962).
20. A. HADJIVASSILIOU and G. BRAWERMAN, *Biochim. biophys. Acta* **103,** 211 (1965).
21. N. OKAMURA and H. BUSCH, *Cancer Res.* **25,** 693 (1965).
22. J. R. TATA, *Nature, Lond.* **219,** 331 (1968).

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Device for controlled drug release—Application to methotrexate infusion in mice

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IN THE COURSE of our research to relate the pharmacokinetics of methotrexate* to its biochemical effect *in vivo*,[1] we developed a small diffusion cell for constant release of this drug in mice. The cell is easily fabricated and implanted subcutaneously. Because it is adaptable for the controlled slow release of numerous other chemicals, we report our technique for its fabrication, its behavior *in vitro*, and plasma concentrations of methotrexate in normal and tumor-bearing mice after its implantation.

Controlled release of drugs from implants has been discussed widely. The most commonly used material for control of release rate is silicone rubber. Folkman and Mark[2] have reviewed the application of this material for release of a variety of drugs and cited a number of examples of sustained pharmacologic effect. Schmidt *et al.*[3] employed silicone rubber capsules for release of the lipophilic antineoplastic drug BCNU.†

* *N*(*p*-[(2,4-diamino-6-pteridinylmethyl)methylamino]benzoyl) glutamic acid, a strong inhibitor of dihydrofolate reductase.

† 1,3-Bis (2-chloroethyl)-1-nitrosourea.

In a series of papers, Garrett and Chemburkar[4–6] discussed drug diffusion through polymeric membranes and examined relevant physicochemical properties and membrane–drug interactions. They reported that silicone membranes were impermeable to phosphate buffer, hydrogen and chloride ions, and the protonated forms of three organic molecules. A recent symposium[7] covered additional developments in the theory and practice of controlled release. A basic problem of polymer diffusion-controlled devices has been a lack of generality; each drug—polymer system exhibits individual properties which influence device design. Data *in vivo* are scarce and often confined to direct biological effects. Balance studies and tissue levels have been reported[8] but generally are restricted to drug-derived radioactivity rather than active species.

Development of cell for methotrexate

Methotrexate (NSC 740) was obtained from the Drug Development Branch of the National Cancer Institute. It was supplied as the free acid, which has a molecular weight of 454. Since its two carboxylic acid groups have p*K*a values of 4·84 and 5·51,[9] it is very soluble at physiologic pH.

Diffusion cells were constructed as illustrated in Fig. 1. Since the steady state Fickian diffusion rate is directly proportional to the area for diffusion and inversely proportional to the length, a wide range of release rates is obtainable by selecting the appropriate geometry. The glass capillary is a section from one of any number of commercially available precision bore micro-pipettes. We have used several that range from 0·27 mm i.d. (area = 0·066 mm^2) to 1·43 mm i.d. (area = 1·61 mm^2); corresponding outside diameters are 1·2 and 2·0 mm. Gel lengths have ranged from 2 to 14 mm. Very short lengths are difficult to control precisely; those of the order of a centimeter are convenient for fabrication and implantation, and do not require excessive times to reach steady state.

In the construction of the cell, a 1% solution of agarose (Indubiose A45, Fisher Scientific Co.) was prepared in 0·9% sodium chloride. This produced a convenient gel which may be stored for long periods

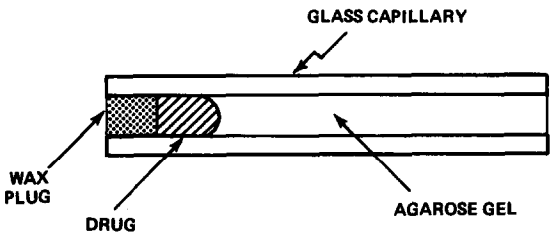

FIG. 1. Schematic diagram of drug diffusion cell.

of time in a refrigerator and then melted by heating. Sections of capillary tubing, lightly flame polished, if desired, were filled by capillarity with the agarose solution, which was stirred continuously on a hot plate; a thick slurry of the drug was then forced into one end. The same end was sealed by pressing it through a shallow layer of wet soft wax. We used Utility Wax Strips (The Hygienic Dental Manufacturing Co., Akron, Ohio) melted into a flat-bottom disposable aluminum dish and then allowed to cool. A small amount of room-temperature-vulcanizing silicone rubber (Silastic Medical Adhesive Tape A, Dow Corning Corp., Midland, Mich.) was placed over the wax in many of the cells to eliminate occasional problems with motion of the wax plug. Evaporative loss from the agarose solution was replaced with distilled water to avoid large shifts in agarose concentration or salt strength. The shortest gel lengths were obtained by making longer sections and then scoring and breaking the glass to the appropriate length.

It is important to avoid any gas bubbles in the drug. Our impression is that bubbles do not appear *de novo* but that small existing gas nucleation sites can grow. In the absence of a gas phase, carbon dioxide generated by the reaction of acid drug with bicarbonate diffuses away from the reaction site rapidly enough to avoid gas problems.

Cell performance in vitro *and* in vivo

Methotrexate in the acid form is only slightly soluble in water. The release rate through a hydrogel is dominated by the influence of basic ions which react with the drug after they have diffused into the cell from its environment. Interstitial fluid contains numerous diffusible basic ions, but the major fraction appears to be bicarbonate. The normal value in human plasma (27 m-equiv/l.)[10] is not greatly different from that in rabbit (28 m-equiv/l.) or rat (21 m-equiv/l.) serum or plasma.[11] We therefore allowed the cells to remain in a solution of 0·25% (30 m-equiv/l.) sodium bicarbonate and 0·9% sodium chloride to approach a steady state release rate. For a gel length of 1 cm and a room temperature of 20–23°, this takes about 3 weeks. The long transient results from the requirement that sufficient bicarbonate must diffuse to the drug to titrate part of it and establish a gradient of methotrexate anion.

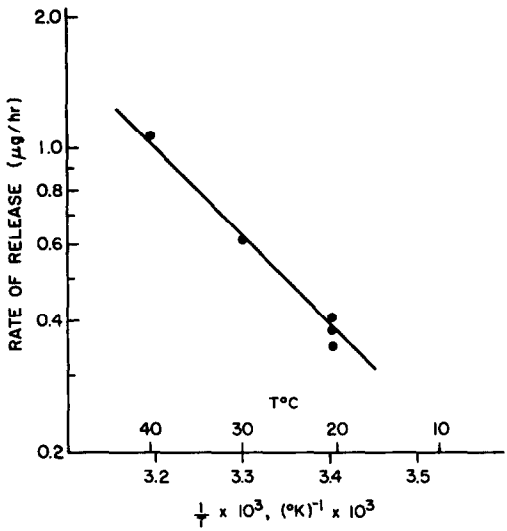

FIG. 2. Steady state release rate from typical cell as function of temperature *in vitro*. Gel length, 9·2 mm; gel cross-sectional area, 0·325 mm^2.

Diffusion from the cell into 0·25% sodium bicarbonate and 0·9% sodium chloride is strongly dependent upon temperature *in vitro*. Figure 2 shows the steady state release rate over the range of physiologic interest as measured by u.v. absorption at 303 nm. A transient period of about 10 hr was observed when the temperature was increased from 21° to 40°. The variation with temperature is significantly greater than would be expected for small ion diffusion through agar gels.[12] This probably results from the reduced

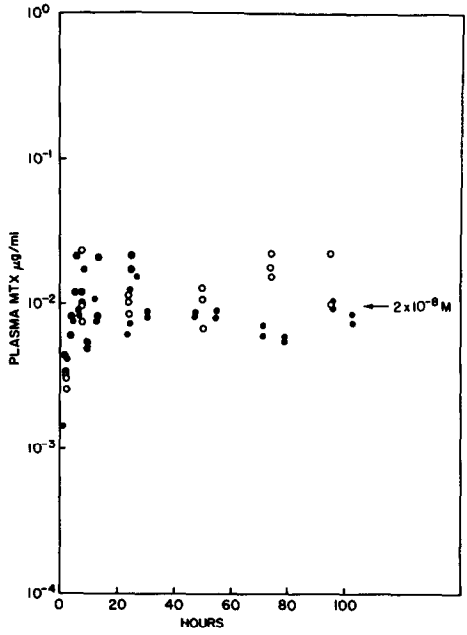

FIG. 3. Plasma concentration of methotrexate in mice after subcutaneous implantation of 0·8 μg/hr cells at time zero. Each point represents a single mouse. Open circles, normal male CDF$_1$ mice; closed circles, male CDF$_1$ mice bearing 0·5 to 1 g Lewis lung tumors.

mobility of methotrexate anion compared with bicarbonate and from a somewhat enhanced role of methotrexate acid solubility at higher temperatures. Rectal temperature of CDF_1 normal mice was 38°; subcutaneous temperature was 1° lower than simultaneously measured rectal temperature immediately after pentobarbital anesthesia.

We implanted cells with predicted release rates of from 0·2 to 9 μg/hr *in vivo* in ether-anesthetized (24 ± 2 g) mice for a variety of toxicologic and biochemical studies. Before implantation, the cells were allowed to approach steady state release rates *in vitro* at room temperature. This permitted measurement of release rates from individual cells or groups of cells before implantation and obviated the long diffusive transient that would otherwise be required. Rates *in vivo* were predicted from those measured *in vitro* on the basis of the temperature dependence (Fig. 2).

The smaller cells were inserted subcutaneously in the back by means of a 13-gauge trocar filled with normal saline; the larger cells were pushed through a small stab wound at the same site.

Plasma concentrations, determined by a sensitive enzyme assay[13] after implantation of approximately 0·8 μg/hr cells, are illustrated in Fig. 3. A nonlinear least squares regression analysis of data from 17 normal and 43 tumor-bearing mice gave a plasma concentration equal to $0{\cdot}0116\,(1 - e^{-0{\cdot}392t})$ μg/ml, where t is the time in hr. The root mean square error is 0·0048 μg/ml, which incorporates all variability of animals, cell release rates, and drug assay. The above correlation indicates that the plasma concentration reaches 90 per cent of the plateau level within 6 hr. A pharmacokinetic model[14] predicted that 90 per cent of the plateau concentration should be achieved within about 7·5 hr with a constant infusion rate of 1 μg/hr. We infer that a steady state release rate is attained quite rapidly *in vivo*, if the cell is allowed to approach steady state in an appropriate buffer before implantation, and that the transient in the plasma concentration is predominantly the expected pharmacokinetic plateau effect when no priming dose is used.

A cell can be removed easily by locating it manually and pushing it through a tiny incision in the skin. Removal results in a rapid decline in plasma concentration.

It is not certain that the rate of release estimated from the studies *in vitro* was identical to that obtained *in vivo*. A pharmacokinetic model[14] overpredicted the observed plateau concentration. This could have resulted from a release rate *in vivo* that was lower than expected because of unanticipated drug instability or other interactions in the subcutaneous environment. It could also have resulted from model deficiencies. The model has been successful in simulating plasma concentrations in mice for 4 hr after intravenous injections at several doses. The model does not include metabolism of MTX to a less reactive or inactive substance with respect to enzyme inhibition, and such metabolism may be significant at very low infusion rates for long times. Metabolism by intestinal bacteria is known to occur in mice.[15,16]

We do not know the maximum length of time that a single cell can maintain a constant plasma concentration in a mouse. Many of the cells were rechecked *in vitro* after removal. There was no significant decrease in rate of release in the absence of visible drug depletion following implantation up to 13 days. It is possible that some long-term tissue reaction could be induced by the device which would reduce drug release; however; we have seen no evidence of this. If drug depletion is the only problem, cells can be made to contain more initial drug. At present, we favor replacement of cells at 2-week intervals for chronic studies.

While we have developed very small diffusion cells for specific studies of methotrexate in mice, the technique should be applicable to other sizes and chemicals. The sizes we have used (0·2 to 9 μg/hr) for methotrexate covers an important biological range. A plasma concentration of 0·01 μg/ml (infusion rate estimated at 0·8 μg/hr) is lethally toxic to some mice if maintained for 50 hr, and to all mice if the cells are left in place. It should be practical to extend the range of release rates in mice about an order of magnitude or so in either direction. Various factors can confound the application of this technique to other chemicals, so the properties of each must be examined carefully. Practical considerations include: drug stability, solubility and dose. Many interactions may occur between drug in the cell and diffusible substances from the interstitial fluid. For many weak acids of limited solubility, the expected release rate is determined by the influx of basic ions from the interstitial fluid and the mobility of drug in the gel. Since mobilities are similar for drug ions of similar size, release rates should be more easily predictable than from polymer-controlled systems. Neutral or basic drugs will be released at rates that are determined by their solubilities and diffusivities in the environment in which they exist and from the dimensions of the gel.

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REFERENCES

1. D. S. ZAHARKO and R. L. DEDRICK, *Proc. Fifth Int. Congr. Pharmac.*, Vol. 3, p. 316. Karger, Basel (1973).
2. J. FOLKMAN and V. H. MARK, *Trans. N.Y. Acad. Sci.* **30,** 1187 (1968).
3. V. SCHMIDT, W. ZAPOL, W. PRENSKY, T. WONDERS, I. WODINSKY and R. KITZ, *Trans. Am. Soc. artif. internal Organs* **18,** 45 (1972).
4. E. R. GARRETT and P. B. CHEMBURKAR, *J. pharm. Sci.* **57,** 944 (1968).
5. E. R. GARRETT and P. B. CHEMBURKAR, *J. pharm. Sci.* **57,** 949 (1968).
6. E. R. GARRETT and P. B. CHEMBURKAR, *J. pharm. Sci.* **57,** 1401 (1968).
7. A. C. TANQUARY and R. E. LACEY (Eds.), *Controlled Release of Biologically Active Agents*, Adv. Exp. Med. Biol. Ser., Plenum Press, New York (in press).
8. G. BENAGIANO, M. ERMINI, C. C. CHANG, K. SUNDARAM and F. A. KINCL, *Acta endocr. Copenh.* **63,** 29 (1970).
9. D. G. LIEGLER, E. S. HENDERSON, M. A. HAHN and V. T. OLIVERIO, *Clin. Pharmac. Ther.* **10,** 849 (1969).
10. W. S. HOFFMAN, *The Biochemistry of Clinical Medicine*, 3rd Edn., p. 8. Year Book Medical Publishers, Chicago (1964).
11. D. S. DITTMER (Ed.) *Blood and Other Body Fluids*, pp. 35–36. Federation of American Societies for Experimental Biology, Washington, D.C. (1961).
12. T. FUJII and H. C. THOMAS, *J. phys. Chem., Ithaca* **62,** 1566 (1958).
13. J. R. BERTINO and G. A. FISCHER, *Meth. med. Res.* **10,** 297 (1964).
14. K. B. BISCHOFF, R. L. DEDRICK, D. S. ZAHARKO and J. A. LONGSTRETH, *J. pharm. Sci.* **60,** 1128 (1971).
15. D. S. ZAHARKO, H. BRUCKNER and V. T. OLIVERIO, *Science, N.Y.* **166,** 887 (1969).
16. D. S. ZAHARKO and V. T. OLIVERIO, *Biochem. Pharmac.* **19,** 2923 (1970).

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Effects of reserpine pretreatment on microsomal enzyme activity*

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THE METABOLISM of reserpine by the liver has long been thought to be largely due to a hydrolytic cleavage involving a non-specific esterase.[1,2] Recently, however, an NADPH, oxygen-dependent mixed function oxidase also has been implicated as an intermediate step in reserpine's biotransformation.[3,4] Since reserpine appears to be a substrate for the microsomal system, it seems reasonable to assume that it may also have the ability to induce microsomal enzymes. This supposition is supported by the observation[5] that, after both large and small doses of reserpine, there is a prominent increase in the amount of hepatic smooth surfaced endoplasmic reticulum. It is the purpose of the present study to describe some of the effects produced by short-term reserpine administration on hepatic microsomal drug metabolism.

Male Wistar rats (175–200 g), albino rabbits (1 to 1·5 kg) and guinea pigs (350–500 g) were used in this study. All animals were maintained on and had free access to food and water. For metabolism studies *in vitro*, animals were sacrificed by stunning followed by exsanguination. Their livers were removed, weighed and homogenized in ice-cold 1·15% KCl. Microsomes were prepared by differential centrifugation according to methods already described.[6] Reaction mixtures of 5-ml vol. were incubated in a Dubnoff metabolic shaker at 37° with air as the gaseous phase. Each 5-ml reaction mixture contained the following constituents: nicotinamide adenine dinucleotide phosphate, 2·0 μmoles; glucose 6-phosphate, 25 μmoles; glucose 6-phosphate dehydrogenase, 2·0 units; nicotinamide, 20 μmoles; magnesium chloride, 25 μmoles; and the microsomes derived from 333 mg liver. The pH of the incubation mixtures was adjusted to 7·4 with 0·1 M phosphate buffer. The pathways studied, the method of assay and the amount of substrate added were: side-chain oxidation of hexobarbital,[7] 3·0 μmoles; and the aromatic hydroxylation of aniline,[8] 10 μmoles. Microsomal protein was determined by the method of Lowry *et al.*[9] using a Technicon auto-analyzer.

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