addition of $10^{-5}M$ DNP further inhibited SCC, but the QCO_2 was significantly increased. This effect is consistent with the action of an uncoupling agent which diminishes ATP production while stimulating metabolism by removing the rate-limiting constraints of respiratory control. The respiratory inhibitors rotenone and antimycin (*9*) also inhibited SCC and QCO_2. In marked contrast to the transport inhibitors, DNP had no significant effect on QCO_2 in the presence of rotenone or antimycin. Although CO_2 may be produced by the pentose shunt pathway, the respiratory quotient of toad bladders is constant (*10*); thus, it is unlikely that changes in the rate of the pentose shunt pathway could account for the present findings.

These results are consistent with the view that respiration which is diminished by direct inhibition of transport can be restimulated by the presence of an uncoupling agent; direct inhibition of respiration by a metabolic inhibitor cannot be reversed by an uncoupling agent. This approach may be useful in the investigation of physiological factors or pharmacological agents that affect biological work functions.

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21 August 1978; revised 17 October 1978

Liposomes and Local Hyperthermia: Selective Delivery of Methotrexate to Heated Tumors

Abstract. *Liposomes with phase transitions a few degrees above physiological temperature delivered more than four times as much methotrexate to murine tumors heated to 42°C as to unheated control tumors. Most of the accumulated drug appeared to be intracellular and bound to dihydrofolate reductase, the enzyme blocked by methotrexate in its role as an antineoplastic agent.*

Systemic drug therapy is a notoriously blunt weapon with which to attack local disease. In a few cases, for instance with antibiotics, a drug may be so selective in action that therapeutic concentrations can be achieved without toxic side effects. More often, a risk of harmful effects must be tolerated. In tumor chemotherapy, for example, the beneficial and toxic actions are so delicately balanced that relatively small degrees of selective drug localization could be useful. We recently suggested a way of combining liposomes as drug carriers with local hyperthermia to achieve preferential local release of drug in a target area (*1*). We now present experiments clearly showing such an effect in vivo.

Liposomes (*2*) are microscopic particles consisting of a single lipid bilayer enclosing a single aqueous compartment (unilamellar vesicle) or a number of concentric bilayers enclosing an equal number of aqueous spaces (multilamellar vesicle). Their use as pharmacological capsules has been limited largely by inability to direct them to particular cells or anatomical sites. Local hyperthermia (*3*), the heating of a region of the body a few degrees above its normal temperature, may provide a basis for such selectivity. Hyperthermia is under study for tumor therapy because it has been reported to affect tumor cells more than normal cells (*4*) and to have synergistic actions in combination with drugs (*5*) and with radiotherapy (*6*).

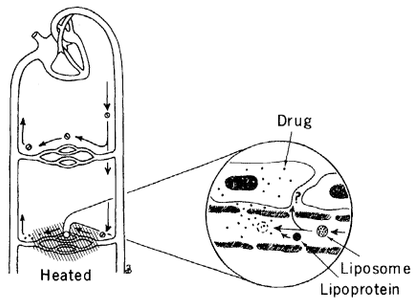

Fig. 1. Schematic view of hypothetical preferential release of liposome contents in a heated area. As liposomes enter the small vessels of the heated region they release their contents at a rate dependent on temperature, the rate of change of temperature, and the action of serum components. Released drug equilibrates throughout the extracellular space and is transported into cells as if injected in free form. As indicated by the question mark, intact liposomes might also pass into the extracellular space through endothelia made leaky by heating, and lipid molecules might be exchanged with cells directly or through the mediation of serum components such as lipoproteins.

Our approach to the combination of liposomes with hyperthermia is shown schematically in Fig. 1 for the case of intravenous injection. Lipids are selected so as to make liposomes with liquid-crystalline phase transition temperatures (T_c) a few degrees above physiological, near the range obtainable by local hyperthermia. The liposomes remain relatively stable in the circulation at temperatures well below T_c but release their contents as T_c is approached (*7*). The rate of release is dependent on the rate of change of temperature and is markedly enhanced by serum components (*1*), principally lipoproteins (*8*). Essentially total release of liposome contents can be achieved in vitro within a few seconds by raising the temperature through T_c in the presence of serum, so it seemed possible that large fractional releases could be achieved in vivo during passage of liposomes through the small arteries, arterioles, and capillaries of a heated region. But it was not clear that conditions of local hyperthermia safe for normal tissues (for example, heating to 42°C for 1 hour) could be exploited effectively. It also seemed possible that the release might take place for the most part in the venous system, from which the drug would simply be washed out of the area by blood flow.

Our aim, therefore, was to develop an experimental system in which the principles of this approach could be studied quantitatively. We chose to use the drug methotrexate (MTX) for three reasons. First, its sodium salt is highly water-soluble and can therefore be encapsulated in and released from liposomes. Second, it acts by binding tightly to a cytoplasmic enzyme, dihydrofolate reductase, with a binding constant of approximately $10^{10}M^{-1}$ (*9*). Therefore, as long as the enzyme's binding capacity is not exceeded, MTX will remain bound in cells long after blood levels have largely dissipated, permitting measurement of total drug de-

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livery to the site. Third, its pharmacology has been studied extensively in mouse tumor systems at both the cellular and the pharmacokinetic levels (*10*), and such background information is very helpful in designing and interpreting experiments. We used low doses of MTX in order to remain in a linear kinetic regime for transport through the tumor cell membrane and to avoid saturation of intracellular binding to dihydrofolate reductase (*11*).

To make liposomes with the appropriate T_c we used a 7 : 3 (by weight) mixture of dipalmitoyl phosphatidylcholine (DPPC: 16-carbon chains; T_c = 41°C by differential scanning calorimetry) and distearoyl phosphatidylcholine (DSPC: 18-carbon chains; T_c = 54°C), both from Avanti Biochemical Co. (Birmingham, Alabama). The lipids gave single spots on thin-layer chromatography of 100-μg samples, both before and after sonication. Tritiated MTX (Amersham [3′,5′,9(n)-^{3}H]MTX) was purified before use by chromatography on DEAE cellulose (*12*). Solutions for encapsulation in liposomes contained 2 percent $NaHCO_3$ and 270 μM MTX with a specific activity of 374 μCi/μmole. The water-soluble fluorophore carboxyfluorescein (CF) was also added to a concentration of 20 mM to permit assessment of the integrity and transition temperature of each liposome preparation.

The lipids were vortex-mixed at 55°C and then sonicated for 1 hour at 55°C under argon (*13*). The sonicate was chilled quickly to 0°C and passed over a 15 by 50 mm column of Sephadex G-25. The resulting liposome suspensions were almost clear initially but became more turbid over a period of hours, indicating slow increase in particle size (*14*). Most experiments were done on the day of preparation, but those performed the following day gave similar results.

After preparation, liposomes were routinely tested for leakage in 10 percent fetal calf serum or mouse plasma by using a method based on fluorescence self-quenching to monitor release of CF during temperature scans (*15*). In the first hour after preparation the temperature of onset of rapid release often shifted from about 38°C to about 40°C and thereafter remained stable. This finding is consistent with a change from small unilamellar vesicles to a form, possibly multilamellar, with more ordered packing of lipids (*14*). More than 90 percent of total encapsulated CF was released within 10 seconds during temperature scans at 14°C per minute. In other experiments CF and [^{3}H]MTX were found to be encapsulated with identical efficiency and to be released from the liposomes at similar rates.

Lewis lung tumors were grown subcutaneously in the flanks of 22- to 27-g $B6D2F_1$ male mice by inoculation of 10^6 viable cells (*16*). Tumors were used on days 6 to 9 and their weights ranged from 75 to 400 mg. They were heated by microwaves (2.45 GHz) from a direct-contact applicator in a system (*17*) that permitted four animals to be heated at once. The temperature in the center of a tumor was maintained at 42.0° ± 0.1°C by the controlling signal from an implanted thermocouple. Temperatures at different points within the tumor varied less than 0.5°C from that at the center. Rectal temperatures, recorded with indwelling probes, generally varied less than 1°C between the time of injection and the end of heating. The mice were anesthetized with chloral hydrate before heating and before they were killed. Tumors were heated to 42°C, and drug was then injected by tail vein. Blood was collected by retro-orbital puncture or, at the time of death, by axillary bleeding. Blood and tumors were burned to CO_2 and H_2O in a Packard Combustor (Downers Grove, Illinois) model 306 for counting of ^{3}H (and sometimes ^{14}C).

We first studied the rate at which [^{3}H]MTX was cleared from the bloodstream when injected in liposomes or in free form (Fig. 2). Most of a dose of free MTX was cleared within 3 minutes by redistribution into extravascular compartments (*11*), whereas liposome-encapsulated MTX remained in the circulation much longer (*18*). Of most interest, the clearance rate of liposomal MTX was increased (65 percent) when a tumor was heated. This suggested rapid release of MTX from the liposomes in the small fraction of the blood passing through the heated area in each circulation. Although some unknown systematic effect of heating on the circulatory system could not conclusively be ruled out, we were encouraged to think that these liposomes would be appropriate for preferential delivery of MTX to heated tumors.

Since liposomal MTX was largely cleared in 1 hour from the circulation of animals with heated tumors (that is, to 10 percent of its initial concentration), we heated for that length of time after injection in subsequent experiments. Figure 3 shows the results of our first experiment

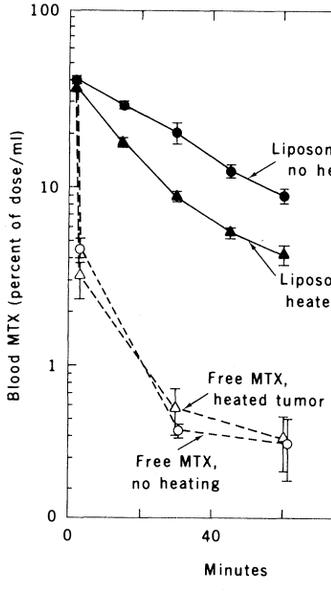

Fig. 2. Clearance from mouse circulation of [^{3}H]MTX injected intravenously as free drug or encapsulated in 7 : 3 DPPC-DSPC liposomes. Heating a subcutaneous tumor at 42°C made no discernible difference in clearance of free drug, which was largely eliminated from the blood by redistribution within the first 3 minutes. Encapsulated MTX cleared with a time constant of 38 minutes in the absence of heating and 23 minutes (for the first 45 minutes) if a tumor was heated. Heating probably acts by releasing drug from the small fraction of liposomes passing through the heated area in each circulation. The MTX doses were 6.0 μg/kg. Liposomes contained 100 mg of lipid per kilogram of mouse body weight. Rectal temperatures averaged 36.0°C and tumors were heated to 42° ± 0.1°C. Each point represents the mean for retro-orbital collections from four animals; error bars represent standard errors. Liposomal MTX was largely in serum, rather than blood cells, after 1 hour.

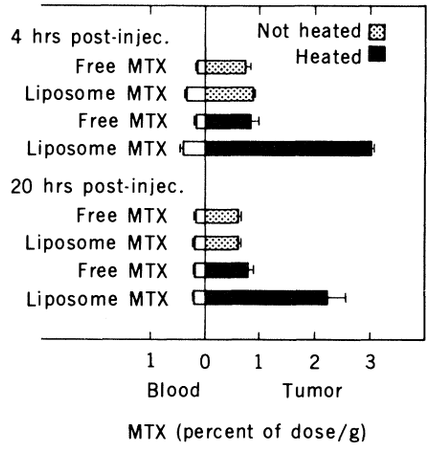

Fig. 3. Incorporation of [^{3}H]MTX into subcutaneously implanted Lewis lung tumors 4 and 20 hours after tail-vein injection of free MTX or liposomes containing MTX. Heated tumors were maintained at 42° ± 0.1°C for about 15 minutes before and 1 hour after injection. At 4 hours heated tumors with liposome MTX had taken up 3.6 times as much MTX as the average of the three control configurations. At 20 hours the factor was 3.4. Blood levels of drug were far too low to account for drug concentrations in the tumors. Doses were (MTX) 4.4 μg/kg and (lipid) ~73 mg/kg. Rectal temperatures averaged 36.5°C. Data are means ± standard errors from four animals.

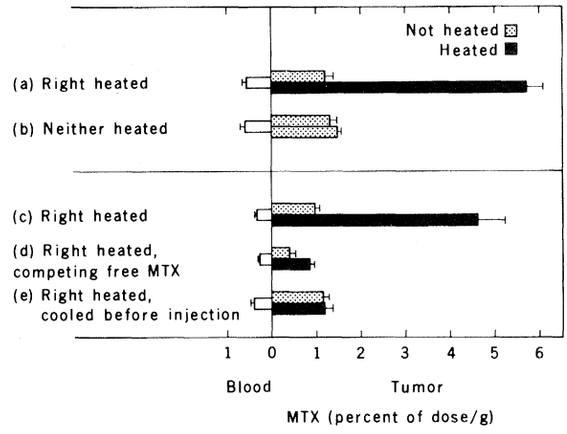

Fig. 4. Incorporation of [^{3}H]MTX in Lewis lung tumors of double-tumor mice 4 hours after tail-vein injection of liposome-encapsulated MTX. Conditions were as in Fig. 3. The dose was (MTX) 6.0 μg/kg and (lipid) 100 mg/kg. Rectal temperatures averaged 35.4°C. (a) Right tumor heated; ratio, 4.8. (b) Neither tumor heated; ratio, 1.15. (c) Right tumor heated; ratio, 4.7. (d) Right tumor heated; 300 mg/kg competing dose of free unlabeled MTX included in injection; uptake in heated tumor decreased more than fivefold, indicating inhibition by unlabeled MTX. (e) Right tumor heated 1 hour, liposomes injected 5 minutes later (after tumors returned to body temperature); ratio, 1.02. Experiments (a) and (b) are separated from (c) to (e) to indicate use of different batches of liposomes and tumors. Data are means ± S.E. from four animals.

on accumulation of [^{3}H]MTX in tumors. The amounts of MTX found 4 hours after injection (3 hours after the end of heating) were approximately the same for the three control groups (free MTX, nonheated tumor; liposome MTX, nonheated tumor; and free MTX, heated tumor), and the amounts of uptake closely matched those obtained for free MTX in previous studies of this tumor system (*11*). In contrast, the amount of [^{3}H]-MTX in the heated tumors treated with liposomes was 3.6 times as great as the average of the controls. As described later, other experiments indicated that essentially all of the ^{3}H counts represented intact MTX and that most of the MTX was intracellular. Blood levels of MTX were far too low to account for the additional accumulation of drug in heated tumors (*19*). After 20 hours there was still considerably more [^{3}H]MTX in the heated, liposome-treated tumors.

To rule out the possibility that differences in uptake resulted from systemic rather than local effects of heating, we did all further experiments on mice carrying tumors in both the left and the right flank (double-tumor mice), one tumor serving in each experiment as an internal control. The large ratios of MTX uptake obtained are indicated in Fig. 4, a and c. The mean ratios obtained for repetitions of this experiment over 3 months with different batches of tumors and of liposomes was 4.3 ± 0.3 (standard error, 21 animals). In the "sham" experiment (Fig. 4b) neither tumor was heated and there was little difference in [^{3}H]MTX uptake.

Persistence of most of the [^{3}H]MTX in the tumors for as long as 20 hours provided strong circumstantial evidence that the drug had penetrated the cells and was bound to dihydrofolate reductase. Since serum levels decline rapidly, it seemed unlike that any other pool would be so persistent. To exclude the possibility that intact liposomes containing [^{3}H]MTX might be sequestered preferentially in the heated tumors, perhaps as a result of endothelial leakiness, we injected [^{3}H]MTX-containing liposomes with a large dose (300 mg/kg) of free, unlabeled MTX. This dose is not acutely toxic to the mice, but it saturates the MTX transport of Lewis lung tumor for about 4 hours and also saturates most of the available MTX-binding capacity of the cells by the fourth hour (*11*). The results are shown in Fig. 4d; uptake was largely inhibited, either at the level of membrane transport or at that of binding to the enzyme. Simple sequestration of liposomes would presumably not have been inhibited.

Figure 4e shows the results when one of the two tumors was heated for 1 hour and then cooled to body temperature before injection of liposomes. This prior heating had little or no effect, indicating that ratios obtained in the standard experiments were not the result of irreversible heat-induced changes in the tumor.

If [^{3}H]MTX is injected intravenously in free form, most of the radioactivity remaining in the circulation after the first 3 hours represents [^{3}H]H_2O (*11*); however, if injected in liposomes, most of the circulating MTX remains intact (*18*). To check whether the tritium counts in the tumors represented intact MTX, we injected drug-containing liposomes, heated for 1 hour, killed the animals at 4 hours, and processed the tumors for high-pressure liquid chromatography (*20*). Virtually all of the ^{3}H counts on the column eluted as MTX, almost none as H_2O, and none as other metabolites or degradation products of MTX.

Other experiments dealt with a number of trivial possibilities: results were similar whether tumors on the right or left were heated; tumor size had little effect on MTX uptake per gram (ratios were similar even when one tumor was several times as large as the other); puncturing the tumors with 25-gauge needles used for inserting thermocouple probes did not change uptake; differences in rectal temperature, from 34° to 37°C, during heating had no major effect, but there was a suggestion that rectal temperatures above 37°C begin to diminish the ratios of heated to unheated uptake; superficial and deep halves of the tumors (both heated and unheated) took up MTX identically; liposomes containing only *p*H buffer had no effect on the uptake of free MTX; and the microwaves had no discernible effect on liposome leakage beyond that produced by equivalent heating under a tungsten-filament lamp.

There are at least six ways in which local hyperthermia might increase the effectiveness of drug-containing liposomes: (i) by promoting selective drug release at temperatures near that of the lipid phase transition of the liposomes; (ii) by increasing the local blood flow; (iii) by increasing endothelial permeability to particles, thereby enhancing accumulation of liposomes in the target tissues; (iv) by increasing the permeability or susceptibility of target cells to drug released from the liposomes; (v) by increasing direct transfer of drug from liposomes to cells—for example, by fusion or endocytosis; and (vi) by decreasing local *p*H and thereby enhancing release from liposomes of anions such as MTX.

Our results are consistent with possibility (i), the hypothesis (see Fig. 1) that local heating leads to release of MTX (with the aid of serum components) and that the released MTX then enters the cells by normal transport mechanisms. We cannot yet rule out (v), a direct liposome-cell interaction rendered temperature-dependent because of changes in the liposomes, the local vascular barrier, or the tumor cells. In principle, several selective processes may be operating simultaneously or may be useful under various sets of conditions. To untangle all of the possibilities will not be easy (*21*). Nevertheless, the preliminary results presented here permit a few additional tentative conclusions about mechanism. The strong circumstantial case for tight intracellular binding of the MTX

to dihydrofolate reductase weighs against (iii) above (unless an initial sequestration were followed by delivery to the cell cytoplasm). Likewise, the ineffectiveness of hyperthermia in promoting uptake of free MTX by the tumor (see Fig. 3) indicates that we are not seeing the results of (iv), a heat-induced increase in cellular transport of MTX. Also, increased blood flow (ii) would not be effective in these experiments since uptake of MTX into Lewis lung tumor cells is limited by membrane transport, not by blood flow (*11*).

The more than fourfold ratio of deliveries is itself potentially useful in therapy, and we have no reason to doubt that higher ratios can be obtained by appropriately optimizing the liposome preparation with respect to size, composition, and charge; by optimizing the temperature of heating; and by combining local hyperthermia with generalized hypothermia to increase the available temperature range. As indicated by other experiments in which the lipid was labeled with [^{14}C]DPPC (*22*), it may also be possible to include lipophilic drugs for selective effects analogous to those seen with the water-soluble MTX.

The major limitation of this approach for cancer chemotherapy is that it does not deal with the problem of widely metastatic disease [unless the reported regression of metastases after heating of primary tumors (*23*) turns out to be a useful phenomenon]. In that limitation it is similar to radiotherapy and to local hyperthermia itself. This approach might, of course, be applied to local lymph nodes in the area of a tumor, to other diseases of better-defined localization (for example, infections), or to widespread diseases of the skin (such as psoriasis), in which heating is feasible and systemic drugs such as MTX are sometimes useful (*24*).

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13. The lipid was initially dried from 2 ml of benzene (with a drop of ethanol added) onto the walls of a glass vial, first with a stream of argon gas and then by several hours on a freeze-drying apparatus. Sonication was done under argon in a closed polystyrene container, using a bath sonicator (Laboratory Supplies, Inc., Hicksville, N.Y.). Less leakage was found below transition if a base other than HCO_3^- was used to titrate MTX.
14. Small unilamellar vesicles are metastable below T_c, and they change into larger forms of a different physical chemical nature [J. Suurkuusk, B. R. Lentz, Y. Barenholz, R. L. Biltonen, T. E. Thompson, *Biochemistry* **15**, 1393 (1976)].
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21. When we tried to assess the role of the phase transition by injecting MTX in liposomes made with DSPC alone, the average heated/unheated ratio was 2.0 ± 0.2 (standard error, 12 mice). But interpretation of the results was difficult: blood levels of encapsulated MTX were still high after 4 hours (liposomes presumably more stable in the circulation); the T_c of liposomes remaining in the serum after 1 hour was found to have decreased toward 42°C (because of slow interaction with serum components); and little of the MTX appeared to be intracellular.
22. When the lipid of DPPC-DSPC liposomes was labeled with [^{14}C]DPPC, heated/unheated ratios averaged 1.9 ± 0.2 (standard error, 11 mice), considerably smaller than that for MTX. This could be explained in a number of ways: direct interaction between liposomes and cells (fusion, endocytosis, or lipid exchange); metabolic alteration of the DPPC (labeled in the fatty acid chains) and uptake in a different form; or disruption of liposomes by serum components and incorporation of the lipid molecules by a secondary process.
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20 December 1978

X-ray Absorption Edge Fine Structure of Potassium Ions in Various Environments: Application to Frog Blood Cells

Abstract. *The edge spectrum is a sensitive probe of local chemistry. Edge spectra of potassium in various chemical environments were measured, and each environment gives a unique edge spectrum. Hydrated potassium has a unique spectrum that is insensitive to counterions. Comparison of the spectra shows that the chemical state of potassium in cells differs appreciably from that in aqueous solutions.*

Diffusible ions play an important role in resting and action potentials, bioenergetics, and other cellular functions. It is therefore of interest to determine their chemical state in the cell. The central question in past investigations on this subject has been whether the majority of intracellular diffusible ions exist in a state of dilute solution (*1*, *2*). Intracellular ions are called free if their chemical state is similar to that in a water solution at equivalent ionic strengths; otherwise they are regarded as bound. The chemical potentials of free and bound ions are expected to be different. Consequently, the thermodynamics of cellu-

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