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DIFFERENTIAL DISTRIBUTION OF LIPOSOME-ENTRAPPED [^{3}H]METHOTREXATE AND LABELLED LIPIDS AFTER INTRAVENOUS INJECTION IN A PRIMATE

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SUMMARY

Positive liposomes consisting of phosphatidylcholine, cholesterol and stearylamine and negatively charged liposomes consisting of phosphatidylcholine, cholesterol and phosphatidylserine, were double labelled with either ^{3}H-labelled dipalmitoyl phosphatidylcholine and [^{14}C]cholesterol or with [^{14}C]cholesterol and [^{3}H]methotrexate entrapped in the aqueous phase. The plasma levels and urinary excretion of radioactivity from sonicated and non-sonicated liposomes were then compared with the levels of radioactivity from free [^{3}H]methotrexate during a 4 h experimental period after an initial intravenous injection in cynomolgous monkeys. Tissue uptake at the completion of the 4 h experimental period was also measured.

It was found that plasma radioactivity from [^{3}H]methotrexate and [^{14}C]-cholesterol in sonicated positive liposomes was cleared more slowly than from comparable non-sonicated liposomes, and considerably slower than from free [^{3}H]-methotrexate. Radioactivity from sonicated negative liposomes was cleared more rapidly than from positive sonicated liposomes. Positive liposomes captured considerably more [^{3}H]methotrexate than negative liposomes and showed very low permeability to [^{3}H]methotrexate in in vitro studies, even in the presence of high concentrations of serum.

[^{14}C]Cholesterol radioactivity was cleared more rapidly from plasma than ^{3}H-radioactivity from liposome-entrapped [^{3}H]methotrexate for double-labelled sonicated liposomes and generally showed greater uptake into tissues and red blood cells. ^{3}H-labelled dipalmitoyl phosphatidylcholine in sonicated positive liposomes was cleared faster than [^{14}C]cholesterol during the first 3 h. The more rapid disappearance of [^{14}C]cholesterol from the plasma was complemented by greater uptake into a number of tissues, and positive non-sonicated liposomes were taken up to a greater extent by the spleen than equivalent sonicated liposomes.

Renal excretion of ^{3}H from liposome-entrapped [^{3}H]methotrexate was considerably less than that of ^{3}H from free [^{3}H]methotrexate. There was insignificant excretion, however, of ^{14}C from cholesterol in the urine.

Entrapment in liposomes completely prevented the otherwise considerable

breakdown of free methotrexate to ^{3}H-containing products in plasma and partially prevented its breakdown in tissues.

These studies indicate marked differences in the distribution of liposomes in vivo due to surface charge and size, and some degree of exchange of the lipid components of the liposome bilayer independent of the distribution of the entrapped species. They also show that entrapment in liposomes can reduce metabolic degradation of a drug, maintain high plasma levels and reduce its renal excretion.

INTRODUCTION

It has now been demonstrated in a number of studies that entrapment of substances inside liposomes can markedly alter their subsequent in vivo distribution. It has been suggested that such a technique might be useful in increasing the effectiveness both of injected drugs [1, 2] ,and of enzymes administered to alleviate the effects of enzyme storage diseases [3, 4]. Increased uptake of liposome-entrapped proteins into the lyzosomes and other intracellular vacuoles of liver cells and other tissues has been demonstrated, which is a promising first step in the latter case [3, 5]. Increased effectiveness of liposome-entrapped drugs and other substances has also been demonstrated in a few cases after injection in vivo [2, 6]. Such entrapment might also be useful in minimizing drug toxicity, which is a continual problem with the cytotoxic drugs used in cancer chemotherapy [7]. Methotrexate is a chemotherapeutic drug which has been used extensively in the past for a variety of malignant disorders, and is currently being successfully used at very high dose levels against osteogenic sarcomas and other tumors [7].

We have found that entrapment of [^{3}H]methotrexate in sonicated, positively charged liposomes markedly reduces the renal excretion of this drug and results in up to 100-fold greater plasma levels of [^{3}H]methotrexate compared to the levels found after intravenous injection of comparable concentrations of free [^{3}H]methotrexate in a cynomolgous monkey (*Macaca irus*) [8]. We have also found that such entrapment completely protects against considerable metabolism of methotrexate in the plasma of this animal and partially protects against such metabolism in tissues [8]. Since methotrexate is rapidly excreted in the urine by what appears to be a renal transport mechanism [9], any methotrexate liberated from disrupted liposomes should be rapidly excreted and/or metabolized. In this paper the distribution of [^{3}H]methotrexate entrapped in the internal aqueous space of both positive and negative liposomes, is compared with the distribution of [^{14}C]cholesterol and ^{3}H-labelled dipalmitoyl phosphatidylcholine incorporated into the lipid bilayers. The quantitatively different distribution of these labelled compounds suggests the possibility of independent exchange reactions for the lipids occurring in vivo, as well as the lack of any renal excretion of intact liposomes.

METHODS AND MATERIALS

Preparation of liposomes. Liposomes were prepared essentially by previously published methods [10, 11]. Usually 64.6 μmol of egg phosphatidylcholine, 45.0 μmol

cholesterol, 10 μCi [^{14}C]cholesterol and 20 μmol stearylamine or 20 μmol bovine brain phosphatidylserine, all as chloroform solutions, were mixed in an all-glass stoppered tube with inlet and outlet tubes provided with stopcocks. The chloroform was removed under vacuum. 1 ml of unlabelled methotrexate solution containing 25 mg methotrexate was evaporated to dryness in a separate tube. This methotrexate was then dissolved in 2 ml of the buffer used for preparing the liposomes; 120 mM NaCl, 4 mM KCl and 20 mM L-histidine, pH 7.40. After adding 80–100 μCi of purified [^{3}H]methotrexate equivalent to 4–5 μg methotrexate, the entire solution was then added to the dried lipids. For all following steps this mixture was kept under a N_2 atmosphere. The mixture in the tube was then mechanically shaken using a Cole-Palmer (Chicago, Ill.) Super Mixer for 15 min, to give non-sonicated liposomes. When sonicated liposomes were used, the mixture was subsequently treated for 1–2 h in a bath type sonicator (Cole-Palmer, 50 W). The tube was positioned for maximum agitation of its contents and the temperature of the bath maintained at approx. 25 °C using a circulating pump. This resulted in considerable clearing of the liposomes which changed from a dense yellow opaque solution to being translucent. Such conditions have previously been shown to result in a large proportion of multilamellar liposomes containing cholesterol being converted to much smaller unilamellar liposomes [11]. The liposomes containing captured [^{3}H]methotrexate plus methotrexate were then separated from the uncaptured methotrexate plus [^{3}H]methotrexate by passing the solution through a Sephadex G-50 (coarse) column (2.2 $\times$ 34 cm). The fractions containing all the lipid phosphorus and the captured [^{3}H]methotrexate eluted first. After elution of non-sonicated liposomes these fractions were turbid and opaque. After the elution of sonicated liposomes these fractions were optically clear. Furthermore, none or at the most 10 % of the preparation was centrifuged down at 100 000 $\times g$ for 1 h suggesting that most of the liposomes had been largely reduced to the much smaller unilamellar type [12]. 10 ml of the eluted fractions showing the greatest capture were pooled and small aliquots were sometimes kept for in vitro permeability studies on [^{3}H]methotrexate efflux from the liposomes in the presence and absence of serum. These efflux studies were performed as previously described [10]. Liposomes containing ^{3}H-labelled dipalmitoyl phosphatidylcholine and [^{14}C]-cholesterol were prepared similarly except that no methotrexate was added and the gel filtration step was omitted. About 20 μCi of ^{3}H-labelled dipalmitoyl phosphatidylcholine was added to the original chloroform liquid mixture at a specific activity of 4 Ci/μmol.

Procedures for injection and sampling on monkeys. The animals were maintained and the experiments were performed essentially as previously described [8, 9]. All animals were cannulated via the right femoral vein and left femoral artery with polyethylene tubing. A size 5 French pediatric feeding tube was then inserted transurethrally into the bladder in order to provide constant urine collection. The samples were injected intravenously over approx. 5 min using a Harvard infusion pump and arterial samples were collected as indicated in Results. For urine collection the bladder was allowed to empty into tubes after the first 10 min and then at 30-min periods. Appropriate "0 time" samples of all of the above were obtained immediately prior to injection.

For sampling of brain tissue after killing of each animal, the skull was rapidly removed and the brain, including brain stem and cerebellum, was dissected free. The total brain was solubilized in 1 M KOH and 1 ml aliquots were taken for counting.

Systemic organ samples were obtained from lung (left diaphragmatic), liver (left lateral), spleen, proximal duodenum, terminal ileum, kidney (cortex and medulla), muscle (biceps longus) and bone marrow (aspirated from the humerus). On all tissue samples, except for bone marrow, gross blood contamination was removed by rinsing three times in 0.9 % NaCl, and then blotting.

Determination of blood volume and blood content of tissues. 33 μCi of ^{125}I-labelled albumin was injected intravenously in a volume of 4.9 ml in 1.8 min at the end of the normal experimental time (240) min as previously described [8]. Blood samples were taken every 5 min for a further 20 min. These showed a constant level of radioactivity. The animal was then sacrificed and tissue samples taken as described above, weighed and counted directly in a Packard 578 gamma scintillation counter.

Measurement of methotrexate concentration by dihydrofolate reductase assay. This determination was performed according to the procedure of Sirotnak and Donsbach [13] in pH 5.8 citrate buffer using crude dihydrofolate reductase from guinea pig liver supernatant [14]. The assay was found to be accurate between 0.5 and 5 ng of methotrexate. Measurements were made on appropriate dilutions of plasma after precipitation of protein by heating at 70–90 °C for 20–30 min. Tissues sample were suspended in 2 ml of normal saline for each gram of tissue, homogenised using a polytron-type tissumizer (Tekmar Co., Cincinnati, Ohio) and heated in a boiling water bath for 10 min. Determinations were made on dilutions of the supernatant after centrifugation. Some plasmas and tissues were also treated with 0.2 % (v/v) Triton X-100 for 20 min before heating and diluting. These various treatments were found to have no effect on the integrity of purified methotrexate.

Preparation of samples for scintillation counting. Separated red blood cells (0.1 ml) were pre-treated with 1 : 1 solution of Protosol (New England Nuclear, Boston, Mass.) and isopropanol at a ratio of 1 ml of the solubilizing mixture for each 0.1 ml of sample. After 1 h of solubilization 0.5 ml of 30 % H_2O_2 was added to decolorize the sample. Plasma (0.1 ml), separated by centrifugation at approx. 2000 rev./min for 10 min in a bench centrifuge, urine (0.1 ml) and cerebro-spinal fluid (0.05 ml) were also pre-treated with 1 ml of Protosol, or water instead could also be added to urine and cerebro-spinal fluid samples to give stable and efficient counting. Tissue samples were solubilized using 1 ml of Protosol for each 100 mg wet weight of tissue and heated at approx. 60 °C for 3 h. After cooling they were sometimes treated with 0.5 ml isopropanol and 0.5 ml 30 % H_2O_2 to decolorize samples such as liver and spleen. All samples treated with Protosol were neutralized with one-tenth volume of 5 M HCl. 10 ml Scintiverse (Fisher Scientific, Rochester, N.Y.) was then added and the samples were counted in a Packard 3330 liquid scintillation counter. Quench correction was obtained using a channels ratio method, or the external standard when the sample counts were low due to considerable quenching.

Materials and animals. Young male or female adult cynomolgous monkeys (*M. irus*, 2.6–4.2 kg), as obtained from commercial suppliers, were used after at least 1 week of stabilization and quarantine. Cannulation procedures were performed following intramuscular administration of anesthetic with appropriate supplemental doses given as needed. They were anesthetized with phencyclidine hydrochloride (2.0 mg/kg, Sernylan; Bio-Ceutic Laboratories) accompanied by 0.1 mg of atropine sulfate.

[3′,5′,9(*n*)-^{3}H]Methotrexate with a specific activity of 9.3 Ci/mmol or 20.5

Ci/g was generously supplied by the Chemical and Drug Procurement Section of the National Cancer Institute through Monsanto Research Corp., Dayton, Ohio. Before use, [^{3}H]methotrexate was purified using the DEAE column chromatography method of Henderson et al. [15]. It was separated into aliquots, lyophilised and stored at −70 °C for up to 3 months. Non-labelled methotrexate was from Lederle Laboratories, Pearl River, N.Y., supplied as a solution prepared for parenteral injections which was stated to be 95 % pure. Phosphatidylcholine was prepared from egg yolks and phosphatidylserine was prepared from beef brain essentially as previously described [11, 16], and was chromatographically pure by silica gel H thin-layer chromatography. The concentration of phospholipid in μmol was measured as the amount of inorganic phosphorus present after $HClO_4$ hydrolysis [16]. Cholesterol was obtained from Sigma Chemical Co., St. Louis, Mo., and crystallized twice from methanol before use. K and K stearylamine (*n*-octodecylamine) was obtained through ICN Pharmaceuticals, Inc., Plainview, N.Y. ^{3}H-labelled dipalmitoyl phosphatidylcholine was a generous gift of Dr. Papahadjopoulos and prepared and purified (90–95 % pure) as previously described [11]. ^{125}I-labelled albumin was from E.R. Squibb and Sons, Inc., New Brunswick, N.J. [4-^{14}C]Cholesterol at a specific activity of 44 Ci/mol and approx. 99 % radiochemical purity was from International Chemical Corp., Irvine, Calif. All other chemicals were at least reagent grade.

RESULTS

Preparation of liposomes containing [^{3}H]methotrexate and their in vitro permeability

Sonicated positively charged liposomes containing phosphatidylcholine, cholesterol and stearylamine were prepared in the presence of [^{3}H]methotrexate, methotrexate and [^{14}C]cholesterol as described in Methods and Materials. Liposomes containing [^{3}H]methotrexate, methotrexate and [^{14}C]cholesterol were separated from unincorporated [^{3}H]methotrexate and methotrexate by gel filtration on a Sephadex G-50 column as shown in Fig. 1A. The first peak contains 72 % of the added lipid phosphorus, 70 % of the added [^{14}C]cholesterol and 5.4 % of the added [^{3}H]methotrexate in fractions 7–12. This resulted in 0.12 % of the added [^{3}H]methotrexate being captured per μmol lipid phosphorus.

Fig. 1B shows the elution pattern of sonicated liposomes prepared in the same way as those shown in Fig. 1A, except that the stearylamine was replaced with 20 μmol of purified bovine brain phosphatidylserine. This confers on the liposomes a net negative rather than a positive charge [17]. Fractions 10–12 contained all the added lipid phosphorus (85 μmol), all the added [^{14}C]cholesterol and 2.06 % of the added [^{3}H]methotrexate. Thus, the 70–72 % recovery of [^{14}C]cholesterol and lipid derived inorganic phosphorus (P_i) for the positively charged, sonicated liposomes might be due to some retention or adsorption of the positively charged liposomes to the Sephadex. This resulted in a capture of 0.027 % [^{3}H]methotrexate per μmol lipid phosphorus. This represented capture of intact methotrexate since subsequent studies on the integrity of liposome-entrapped methotrexate in the plasma, showed 90–100 % of the ^{3}H-radioactivity represented intact methotrexate. Since methotrexate is negatively charged at pH 7.4 the 3.8-fold greater capture per μmol phosphatidylcholine for positively charged liposomes is likely to be due to charge attraction. Non-sonicated positively charged liposomes containing [^{14}C]cholesterol and [^{3}H]methotrexate and

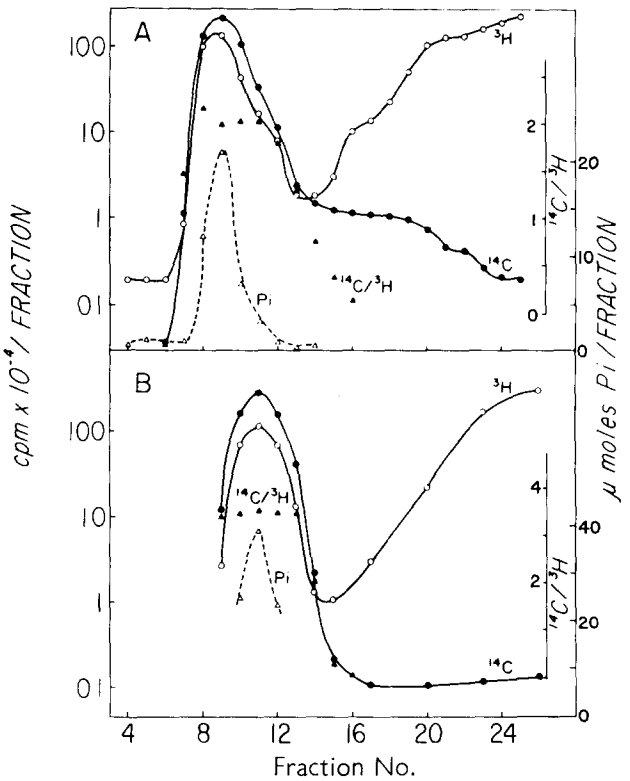

Fig. 1. Separation of liposome-entrapped [^{3}H]methotrexate from free [^{3}H]methotrexate. (A) Liposomes consisting of 64.6 μmol egg phosphatidylcholine, 45 μmol cholesterol, 10 μCi [^{14}C]cholesterol and 19.5 μmol stearylamine were mixed and sonicated in 2 ml of buffer containing 25 mg methotrexate and approx. 100 μCi [^{3}H]methotrexate as described in Methods and Materials. The faintly translucent suspension was then eluted on a Sephadex G-50 column and 5-ml fractions were collected as described in Methods and Materials. (B) Liposomes were sonicated and eluted on a Sephadex G-50 column as described in A, except they contained 20 μmol bovine brain phosphatidylserine instead of stearylamine.

sonicated and non-sonicated positively charged liposomes containing [^{3}H]methotrexate as the only radioactive component, were also prepared. Positively charged non-sonicated liposomes captured about twice as much as positively charged sonicated liposomes [8].

In order to assess the probable stability of the liposome-entrapped methotrexate once it was injected intravenously, in vitro permeability studies were performed by dialysis, as previously described [10] in the presence and absence of varying amounts of human serum albumin inside the dialysis bags. The dialysis bags were placed into test tubes containing the dialysate immediately after all additions had been made, omitting any preliminary bulk dialysis. The results of these experiments are shown in Table I. As can be seen, very little methotrexate leaked out of the liposomes even in the presence of 60 % serum. The non-sonicated liposomes showed some increase in permeability rates in the presence of serum albumin, whereas much less effect was seen for the sonicated liposomes. Control studies (see Table I) showed that free methotrexate in the presence or absence of serum albumin leaked out of the dialysis bags at a significantly higher rate.

TABLE I

PERMEABILITY OF STEARYLAMINE LIPOSOMES TO [^{3}H]METHOTREXATE IN VITRO

Permeability of free methotrexate or methotrexate entrapped in liposomes. This was measured using the dialysis technique described in Methods and Materials. Percent of total refers to the [^{3}H]methotrexate diffusing in 1 h as a percentage of the amount present at the start of the hour period.

Type of liposome	Time (h)	Percent of total [^{3}H]methotrexate diffusing per h in presence of:			
		No serum	20 % serum	40 % serum	60 % serum†
Sonicated*	1	0.97	1.11	1.32	0.95
	2	0.43	0.55	0.69	0.63
	3	0.27	0.37	0.49	0.45
	4	0.21	0.28	0.38	0.36
Non-sonicated*	1	0.97	2.16	1.77	1.93
	2	0.44	1.28	1.21	1.30
	3	0.23	0.66	0.72	0.70
	4	0.14	0.43	0.47	0.80
Free [^{3}H]methotrexate**	1	40.8			17.6
	2	29.3			11.1
	3	17.6			7.4
	4	15.0			–

* Average of two determinations.
** Average of four determinations.
† Human serum albumin.

Plasma levels of radioactivity

Fig. 2 compares the plasma levels of ^{3}H-radioactivity from [^{3}H]methotrexate after entrapment in sonicated and non-sonicated positive liposomes with plasma levels after injection of a comparable amount of free methotrexate plus approx. 100 μCi [^{3}H]methotrexate. No [^{14}C]cholesterol was present in these liposomes. As can be seen entrapment in liposomes results in a marked increase in the retention of ^{3}H radioactivity in the plasma, especially with sonicated liposomes. After 240 min there was an approx. 100-fold greater level of ^{3}H radioactivity after injection of [^{3}H]-methotrexate entrapped in sonicated liposomes compared to the free drug. The early rapid disappearance phases for the free drug are much reduced or absent for [^{3}H]-methotrexate entrapped in sonicated liposomes. The terminal linear phases gave a $t_{\frac{1}{2}}$ of 14.7 h for [^{3}H]methotrexate in sonicated liposomes, 10.5 h for [^{3}H]methotrexate in non-sonicated liposomes and 2.7 h for free [^{3}H]methotrexate.

Fig. 3 shows the plasma levels of ^{14}C radioactivity from [^{14}C]cholesterol and ^{3}H radioactivity from [^{3}H]methotrexate after injection of [^{3}H]methotrexate entrapped in positively charged sonicated and non-sonicated liposomes, compared with the plasma levels of ^{3}H radioactivity after intravenous injection of free [^{3}H]methotrexate. Again the [^{3}H]methotrexate levels are some 5-fold higher in sonicated liposomes than non-sonicated liposomes and in this case about 50-fold higher than the [^{3}H]methotrexate levels found after injection of two different concentrations of the free drug. In these experiments in contrast to those shown in Fig. 2, the amount of free drug injected was 14–86 times greater than the liposome-entrapped drug, but this resulted

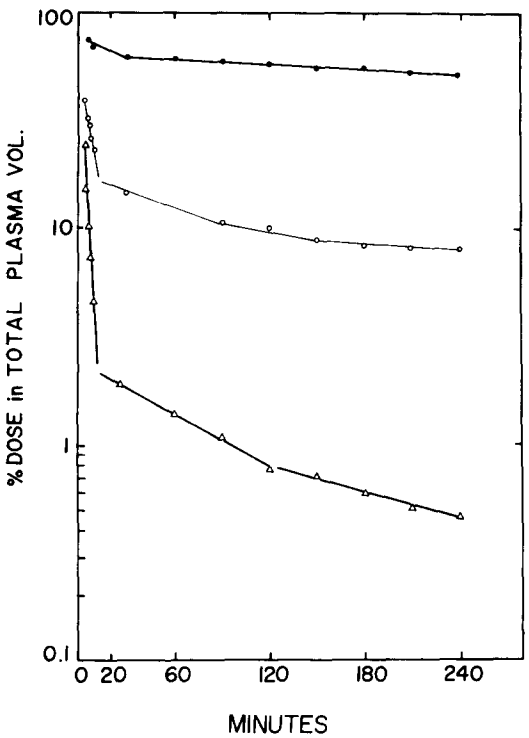

Fig. 2. ^{3}H radioactivity levels in plasma after intravenous injection of liposome-entrapped [^{3}H]-methotrexate and free [^{3}H]methotrexate. ●, [^{3}H]methotrexate in sonicated liposomes; average of three separate experiments with 0.88 mg methotrexate plus 4 μCi [^{3}H]methotrexate injected; average monkey weight was 3.8 kg. ○, non-sonicated liposomes containing 2.2 mg methotrexate plus 10 μCi [^{3}H]methotrexate, average of two experiments; average monkey weight was 3.9 kg. △, free methotrexate, 1.10 mg methotrexate plus 100 μCi [^{3}H]methotrexate was injected into a 3.0 kg monkey. The total plasma volume was calculated on the basis of 42.7 ml plasma/kg, determined as the average plasma volume using ^{125}I-labeled albumin [8]. 10-ml volumes were injected intravenously over 4.9 min as described in Methods and Materials. No [^{14}C]cholesterol was present in either liposome preparation.

in only a small increase in the percentage of the dose retained. The free drug shows the same triphasic pattern of disappearance as seen in Fig. 2 and this is not seen for the liposome-entrapped drug. In the case of the sonicated liposomes the [^{14}C]cholesterol was removed at an approx. 2-fold faster rate than the [^{3}H]methotrexate entrapped in the aqueous phase.

Fig. 4 shows that the radioactivity from [^{3}H]methotrexate and [^{14}C]cholesterol in sonicated negatively charged liposomes are both removed more rapidly than in sonicated positively charged liposomes (compare Figs. 3 and 4). ^{3}H from ^{3}H-labelled dipalmitoyl phosphatidylcholine in the positive, sonicated ^{3}H-labelled dipalmitoyl phosphatidylcholine/[^{14}C]cholesterol liposomes seems to be removed more rapidly than [^{14}C]cholesterol during the first 3 h. For comparison the plasma levels of ^{3}H after injection of free [^{3}H]methotrexate with 53.2 mg methotrexate is also shown. The levels found and the apparent triphasic disappearance curve is similar to that

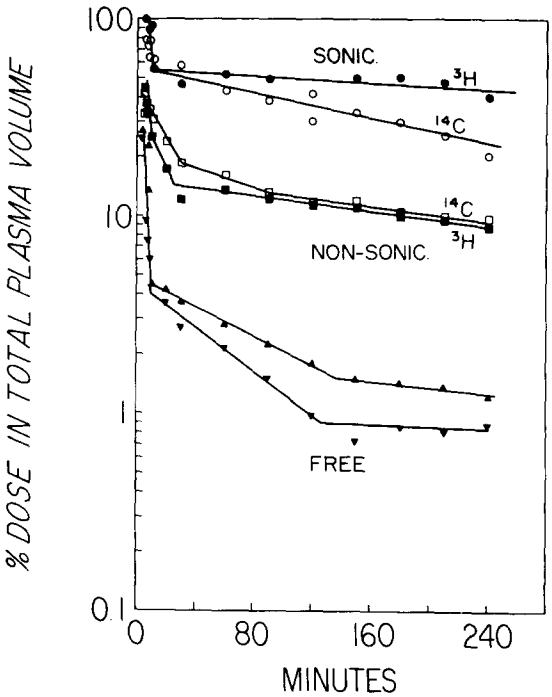

Fig. 3. Radioactivity levels in plasma after intravenous injection of liposome-entrapped [^{3}H]methotrexate plus [^{14}C]cholesterol or free [^{3}H]methotrexate. ●, ○, 10 ml of sonicated positively charged liposomes containing 1.34 mg methotrexate (5.4 % of the added methotrexate) and 7.8 μCi [^{14}C]-cholesterol were injected intravenously into a 3.74 kg monkey over 4.9 min. ●, ^{3}H radioactivity; ○, ^{14}C radioactivity. ■, □, 10 ml of non-sonicated positively charged liposomes containing 1.29 mg methotrexate (5.2 % added methotrexate but about half the added lipid) and 5.1 μCi [^{14}C]cholesterol were injected intravenously into a 3.12 kg monkey over 4.9 min. ■, ^{3}H radioactivity; □, ^{14}C radioactivity. ▲, 111.3 mg free methotrexate and approx. 100 μCi [^{3}H]methotrexate in 10 ml volume was injected intravenously into a 4.42 kg monkey over 4.9 min. ▼, 5 ml containing 19.4 mg free methotrexate and approx. 100 μCi [^{3}H]methotrexate was injected intravenously into a 3.88 kg monkey over 4.9 min. The results are shown as the percentage of the total injected dose of [^{3}H]methotrexate or [^{14}C]cholesterol present in the total plasma compartment at the indicated times.

found for the different amounts of injected free [^{3}H]methotrexate and methotrexate shown in Figs. 2 and 3. The negative sonicated liposomes appear to show a biphasic rate of disappearance, while the positive sonicated ^{3}H-labelled dipalmitoyl phosphatidylcholine/[^{14}C]cholesterol liposomes show an irregular pattern in this case.

Integrity of methotrexate in plasma

Previously we have found considerable metabolism of [^{3}H]methotrexate to non-methotrexate products containing ^{3}H in the plasma of cynomolgous monkeys [8]. This was also found for the present series of experiments, as shown in Table II, by comparing the amount of methotrexate indicated by the dihydrofolate reductase assay with the amount of methotrexate calculated from the level of radioactivity. As reported previously [8], it can be seen that entrapment of methotrexate in liposomes essentially completely protects against this breakdown of methotrexate in the

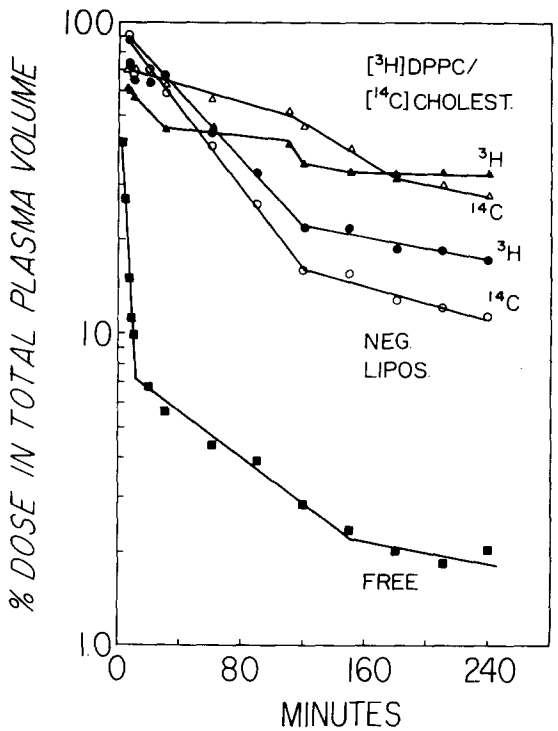

Fig. 4. Radioactivity levels in plasma after intravenous injection of liposome-entrapped [^{3}H]methotrexate plus [^{14}C]cholesterol, ^{3}H-labelled dipalmitoyl phosphatidylcholine plus [^{14}C]cholesterol or free [^{3}H]methotrexate. ▲, △, sonicated, positively charged liposomes labelled with ^{3}H-labelled dipalmitoyl phosphatidylcholine and [^{14}C]cholesterol (see Methods and Materials). 10 ml injected intravenously into a 3.0 kg monkey. ▲, ^{3}H; △, ^{14}C. ●, ○, sonicated negatively charged liposomes containing [^{14}C]cholesterol and entrapped [^{3}H]methotrexate plus 0.28 mg cold methotrexate. 9.7 ml injected in 4.8 min into a 3.18 kg monkey. ●, ^{3}H; ○, ^{14}C. ■, 5 ml containing 53.2 mg free methotrexate plus approx. 100 μCi [^{3}H]methotrexate was injected intravenously in 4.9 min into a 4.14 kg monkey.

plasma. Thus, the ^{3}H radioactivity shown in Figs. 2–4 represents mainly intact methotrexate for the liposome-entrapped drug, but only a small fraction of the total ^{3}H radioactivity represents intact methotrexate after injection of the free drug.

Uptake of [^{3}H]methotrexate and [^{14}C]cholesterol by red blood cells

Fig. 5 shows the uptake of ^{14}C and ^{3}H radioactivity by washed red blood cells. There seems to be a marked initial uptake of both ^{14}C and ^{3}H after injection of positively charged non-sonicated liposomes, which is not seen for positively charged sonicated liposomes also containing [^{3}H]methotrexate and [^{14}C]cholesterol. Both types of liposomes, however, show a long term uptake of ^{14}C compared to ^{3}H, which is greater in the case of the sonicated liposomes. Thus, for sonicated liposomes about 10 % of the total dose of ^{14}C is present in the red blood cells, compared to about 25 % in the plasma (see Fig. 3) at 4 h. The corresponding figures for ^{3}H are 7 and 45 %. Very little uptake of ^{3}H by red blood cells from non-sonicated liposomes is seen at 4 h. Because of its transient nature the initial uptake of non-sonicated liposomes may be due to reversible binding.

TABLE II

BREAKDOWN OF FREE AND LIPOSOME-ENTRAPPED METHOTREXATE IN PLASMA

Intact methotrexate was measured by the dihydrofolate reductase assay and is expressed as a percentage of the methotrexate calculated from ^{3}H radioactivity levels, assuming no breakdown of methotrexate. Details of the dihydrofolate reductase assay and Triton treatment are described in Methods and Materials. Negative liposomes are as used in the experiment in Fig. 4 and sonicated positive liposomes are as used in Fig. 3. Each value is the average of three determinations on the same sample, which did not differ by more than 10 %.

Time (min)	Free methotrexate (5.01 mg/kg)	Percent intact methotrexate			
		Liposome-entrapped methotrexate			
		Sonicated negative liposomes		Sonicated positive liposomes	
		−Triton	+Triton	−Triton	+Triton
4	97	–	–		
20	64	82	100		
30	39	89	–		
120	11	117	–		
180	9	86	–		
240	5	71	107	37	100

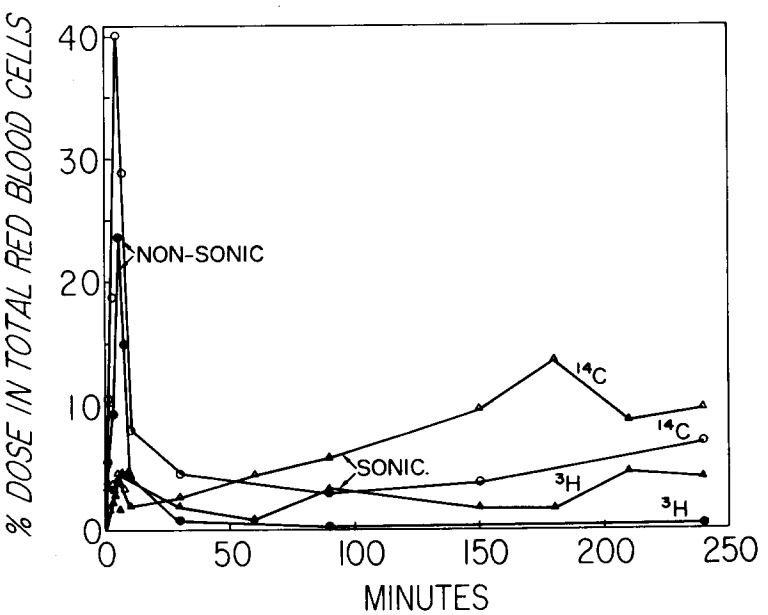

Fig. 5. Uptake of ^{14}C and ^{3}H by washed red blood cells after injection of liposomes containing [^{3}H]methotrexate and [^{14}C]cholesterol. Red blood cells were separated from plasma by centrifugation at 2000 rev./min for 10 min and washed 3 × in 30 vol of 0.9 % saline. The final pellet was dispersed to a known volume and aliquots prepared and counted as described in Methods and Materials. The total volume of red blood cells was obtained from the calculated total plasma volume and the hematocrit value. ●, ○, non-sonicated positively charged liposomes. Identical experiment to that shown in Fig. 3. ●, ^{3}H; ○, ^{14}C. ▲, △, sonicated positively charged liposomes. Identical experiment to that shown in Fig. 3. ▲, ^{3}H; △, ^{14}C.

Uptake by tissues

The tissue uptake at 4 h for sonicated positive and negative liposomes is shown in Table III. All these values have been corrected for ^{3}H and ^{14}C present due to plasma. The plasma content without prior exsanguination can range from 1 %/g for brain to 24 %/g for liver [8]. In comparing uptake for the different types of liposomes probably only large differences are significant due to the marked variation found from animal to animal. Thus, although radioactivity from both [^{3}H]methotrexate and [^{14}C]cholesterol in sonicated, negative liposomes are generally taken up to the same extent or to a greater extent compared to sonicated positive liposomes, there appears to be a marked reduction in uptake by the spleen and bone marrow of ^{3}H and ^{14}C from negative liposomes. In the case of liver and spleen this uptake of ^{3}H radioactivity represented 29 and 66 % intact methotrexate, respectively, for the sonicated, positive liposomes. There was a negligible effect of Triton on this enzymatic determination of intact methotrexate. There is clearly a definite increased uptake of ^{14}C from [^{14}C]-cholesterol relative to ^{3}H from [^{3}H]methotrexate for a number of tissues. The amount of this ^{14}C which represented unaltered cholesterol was not, however, determined. Any breakdown of cholesterol, however, is likely to be subsequent to its uptake.

Table IV shows the uptake of ^{3}H and ^{14}C from sonicated, positive liposomes containing ^{3}H-labelled dipalmitoyl phosphatidylcholine and [^{14}C]cholesterol and non-sonicated positive liposome scontaining [^{3}H]methotrexate and [^{14}C]cholesterol. For the non-sonicated positive liposomes there was a marked increase in uptake by

TABLE III

TISSUE UPTAKE AT 4 h

Values are percentage dose/g. Sonicated positive liposome results are for duplicate determinations on two separate animals and are therefore the mean ±S.E. where $n = 4$. For the sonicated, negative liposomes the results are for duplicate determinations on tissues from a single animal, which generally did not differ by more than 10 %. For experimental details see Figs. 3 and 4. Corrected for plasma content.

	[^{3}H]Methotrexate/[^{14}C]cholesterol in sonicated positive liposomes			[^{3}H]Methotrexate/[^{14}C]cholesterol in sonicated negative liposomes	
	^{3}H	^{14}C	Intact methotrexate (%)	^{3}H	^{14}C
Liver	0.31 ±0.02	0.48 ±0.02	29	0.43	0.63
Spleen	0.50 ±0.04	0.56 ±0.029	66	0.070	0.31
Kidney	0.023±0.007	0.021±0.002		0.036	0.035
Ileum	0.013±0.002	0.016±0.005		0.039	0.020
Duodenum	0.023±0.002	0.019±0.003		0.019	0.015
Lung	0.009±0.003	0.065±0.009		0.032	0.094
Bone marrow	0.057±0.012	0.100±0.01		0.024	0.010
Bile (per ml)	0.30 ±0.01	0.20 ±0.015		n.d.	n.d.
Muscle	0.006±0.004	0.003±0.0001		n.d.	n.d.
Brain	0.012	0.031		0.002	0.006

n.d., not determined

TABLE IV

TISSUE UPTAKE AT 4 h

Data represent average of results in percentage dose/g from duplicate determinations in a single animal in each case. For experimental details see Figs. 3 and 4. Corrected for plasma content.

Tissue	^{3}H-labelled dipalmitoyl phosphatidylcholine/[^{14}C]-cholesterol in sonicated positive liposomes		[^{3}H]Methotrexate/[^{14}C]cholesterol in non-sonicated positive liposomes		
	^{3}H	^{14}C	^{3}H	^{14}C	Intact methotrexate (%)
Liver	0.15	0.31	0.17	0.48	20
Spleen	0.14	0.36	1.02	3.03	22
Kidney	0.013	0.001	0.029	0.022	
Ileum	0.007	0.006	0.018	0.017	
Duodenum	0.015	0.011	0.035	0.017	
Lung	n.d.	0.022	0.011	0.073	
Bone marrow	n.d.	n.d.	0.013	0.028	
Bile (per ml)	n.d.	n.d.	2.21	0.54	
Brain	n.d.	n.d.	not detectable	0.023	

n.d., not determined.

TABLE V

^{14}C/^{3}H RATIO FOR TISSUE UPTAKE NORMALIZED TO ORIGINAL LIPOSOME RATIO

Ratios are normalised by setting original liposome ratio = 1.0 (actual ratio shown in last row), so that they can be simply obtained by dividing respective percentage dosage for ^{14}C by ^{3}H from Tables III and IV.

Tissue	Sonicated, positive [^{14}C]cholesterol/ ^{3}H-labelled dipalmitoyl phosphatidylcholine liposomes	Sonicated, positive [^{14}C]cholesterol/ [^{3}H]methotrexate liposomes	Sonicated, negative [^{14}C]cholesterol/ [^{3}H]methotrexate liposomes	Non-sonicated positive [^{14}C]-cholesterol/ [^{3}H]metho-trexate liposomes
Liver	1.72	1.55	1.47	2.67
Spleen	2.57	1.12	4.43	2.97
Kidney	0.077	0.91	0.97	0.76
Ileum	0.86	1.23	0.51	0.94
Duodenum	0.73	0.82	0.79	0.49
Lung	only ^{14}C uptake	7.22	2.94	6.64
Bone marrow	n.d.	1.75	0.42	2.15
Bile	n.d.	0.67	n.d.	0.24
Muscle	n.d.	0.50	n.d.	n.d.
Brain	n.d.	2.58	3.0	only ^{14}C uptake
Original liposomes	1.56	2.18	2.76	1.33

n.d., not determined.

TABLE VI

TISSUE UPTAKE OF FREE [^{3}H]METHOTREXATE 4 h AFTER INTRAVENOUS INJECTION

Corrected for plasma content, averaged from 0.0024 % /g for liver to an average 0.0008 % dose/g for seven different brain regions. Determinations of intact methotrexate were by the dihydrofolate reductase assay.

Tissue	Dose: 25.1 mg/kg		12.86 mg/kg		5.01 mg/kg
	Percent dose/g	Percent intact methotrexate	Percent dose/g	Percent intact methotrexate	Percent dose/g
Liver	0.012	6.2	0.028	4.45	0.036
Spleen	0.0017	3.0	0.0051	7.8	0.0043
Kidney	0.011	n.d.	0.033	26	0.012
Ileum	0.010	n.d.	0.0067	2.0	0.011
Duodenum	0.0036	n.d.	0.0068	n.d.	0.0042
Lung	0.0013	n.d.	0.0044	n.d.	0.0029
Brain	0.0026	n.d.	0.0043	n.d.	0.0020
Bone marrow	n.d.	n.d.	0.0031	12.5	0.0027

n.d., not determined.

the spleen and again uptake of ^{14}C was greater, in some cases by several-fold, than uptake of ^{3}H. The amount of radioactivity that represented intact methotrexate in liver and spleen, 20 and 22 %, respectively, was lower than for sonicated positive liposomes expecially in the case of the spleen. Again, Triton X-100 had no significant effect on these determinations.

Table V shows the actual ^{14}C/^{3}H ratios, corrected for plasma content, expressed relative to the ratio of the original liposomes which is equivalent to setting this ratio at unity. This allows ready appreciation of the relative amounts of ^{14}C and ^{3}H taken up. The actual original ratios for the liposomes varied from 1.56 to 2.76 and are also shown in Table V. Certain tissues such as lung, brain and spleen, clearly showed marked preferential uptake of ^{14}C.

To permit comparison of the uptake of ^{3}H from liposome-entrapped [^{3}H]-methotrexate with ^{3}H from free [^{3}H]methotrexate, Table VI shows the uptake of ^{3}H from free [^{3}H]methotrexate at various dose levels of methotrexate. The amount of the radioactivity which represents intact methotrexate is also shown for several tissues, emphasizing the fact that ^{3}H cannot be equated with intact methotrexate. There seems to be no clear relationship between size of dose and percentage uptake.

By comparing the data for uptake of ^{3}H from free [^{3}H]methotrexate with that shown in Tables III and IV for liposome-entrapped [^{3}H]methotrexate it can be seen that many tissues take up considerably more ^{3}H from liposome-entrapped [^{3}H]-methotrexate compared to ^{3}H from free [^{3}H]methotrexate. There is a 10-fold or more increased uptake of ^{3}H from liposome-entrapped [^{3}H]methotrexate in the case of liver and spleen. For spleen this increased uptake is up to $2 \cdot 10^2$–$20 \cdot 10^2$-fold greater for non-sonicated liposomes in terms of radioactivity. This ratio is even larger if based on actual intact methotrexate since the percentage of intact methotrexate taken up is greater for liposome-entrapped methotrexate. The percentage of intact methotrexate is also low for free methotrexate taken up by a number of other issues. The level of

TABLE VII

RENAL EXCRETION

All are positively charged liposome experiments, prepared as described in Methods and Materials and from the same experiments described in Figs. 3 and 4. For free [^{3}H]methotrexate the results shown are an average of four separate experiments, and approx. 100 μCi of free [^{3}H]methotrexate with 5–25 mg methotrexate/kg was injected intravenously. Urine was collected over the indicated time periods as described in Methods and Materials.

Time	Percent dose/ml						
	Sonicated ^{3}H-labelled dipalmitoyl phosphatidylcholine/ [^{14}C]cholesterol		Sonicated [^{3}H]methotrexate/ [^{14}C]cholesterol		Non-sonicated [^{3}H]methotrexate/ [^{14}C]cholesterol		Free [^{3}H]methotrexate
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	
10	0.17	0.001	0.075	0.005	0.30	0.001	1.46
30	1.11	0.01	0.21	0.001	2.07	0.004	1.52
60	0.96	0.01	0.36	0.003	1.74	0.007	3.19
90	0.70	0.02	0.39	0.005	1.59	0.006	1.46
120	0.67	0.02	0.37	0.007	1.02	0.005	1.05
150	0.68	0.03	0.35	0.006	0.98	0.006	0.65
180	0.70	0.04	0.26	0.006	0.83	0.009	0.65
210	0.55	0.05	0.34	0.010	0.84	0.010	0.79
240	0.46	0.05	0.33	0.012	1.09	0.016	0.32
Average percentage dose/ml	0.67	0.025	0.29	0.006	1.16	0.007	1.23
±S.E.	±0.09	±0.006	±0.03	±0.001	±0.18	±0.001	±0.28

intact methotrexate in kidney after injection of free drug is, however, relatively high which is consistent with the appreciably lower breakdown found in urine compared to plasma (unpublished observations). Lung also takes up a relatively high amount of ^{3}H from liposome-entrapped [^{3}H]methotrexate compared to free [^{3}H]methotrexate. The differences in uptake of ^{3}H from free and liposome-entrapped [^{3}H]methotrexate by other tissues is somewhat less, though dependent on the type of liposome.

Renal excretion

As shown in Table VII entrapment of [^{3}H]methotrexate in sonicated positively charged liposomes causes a 4-fold reduction in renal excretion of ^{3}H ,whereas for positive non-sonicated liposomes no significant effect was seen. This effect has been noted previously [8] and because of the high plasma levels maintained, translates into a marked reduction in renal clearance. It can now be seen that although ^{3}H from ^{3}H-labelled dipalmitoyl phosphatidylcholine and [^{3}H]methotrexate are excreted in comparable amounts, there is insignificant excretion of [^{14}C]cholesterol.

DISCUSSION

Liposome-entrapped [^{3}H]methotrexate and [^{14}C]cholesterol in plasma

Our results on the disappearance from plasma of ^{3}H from liposome-entrapped [^{3}H]methotrexate and its uptake into tissues are in general agreement with those of

other workers using smaller experimental animals and other entrapped substances. Most of the previous work apparently also used large non-sonicated liposomes or liposomes sonicated for a very brief period, although Gregoriadis et al. [18] have noted that their preparations are almost clear after 10 s of sonication. The decreased disappearance of ^{3}H from liposome-entrapped [^{3}H]methotrexate from plasma is similar to that reported by others for several liposome-entrapped compounds [1, 19–22], including a preliminary report on methotrexate entrapped in negatively charged, non-sonicated liposomes in rats [23]. It has also been reported that the rate of disappearance of positively charged liposomes from plasma is slower than that of neutral or negatively charged liposomes [1, 19], and small liposomes disappear more slowly than large liposomes in rats [19]. This study shows that with a substance like methotrexate which is rapidly excreted in the free form and shows no appreciable leakage out of liposomes, greater than 50-fold increases in plasma levels can be obtained for the liposome-entrapped drug compared to the free drug. In view of the fact that the free, but not the liposome-entrapped drug, is extensively metabolised the increased level in terms of intact methotrexate is even greater (up to 10^{3}-fold). Unlike penicillin [1], actinomycin D [1, 21] and univalent cations in the presence of serum [22], the permeability of liposomes to methotrexate entrapped in the internal aqueous volume remains low even in the presence of high concentrations of serum.

The in vitro permeability data, the lack of metabolism of methotrexate and the rapid removal of free methotrexate suggests that all the ^{3}H radioactivity detected in the plasma compartment represents [^{3}H]methotrexate plus methotrexate entrapped in liposomes. In view of this, the increased rate of removal of [^{14}C]cholesterol and [^{3}H] dipalmitoyl phosphatidylcholine [8] at least for sonicated liposomes, suggests preferential removal of cholesterol and phospholipid by exchange process(es) which does not affect the basic structural integrity and permeability properties of the liposomes. This selective exchange of cholesterol is supported by the fact that a large number of tissues take up more ^{14}C than ^{3}H from both sonicated and non-sonicated liposomes, although this could also result from leaky fusion (refs. 26–28 and Fig. 6B). This uptake would presumably be initially into the plasma membranes of tissue cells. There also appears to be selective uptake of ^{14}C into the red blood cells which is greatest for sonicated liposomes at longer time periods (see Fig. 5), as well as an initial rapid and reversible binding of positive non-sonicated liposomes to red blood cells. Rapid exchange of [^{14}C]cholesterol from phosphatidylcholine/cholesterol liposomes both with other liposomes and mitochondria where 50 % of the cholesterol exchange within 1 h and which was not dependent on an exchange protein, has been reported [24], as well as exchange of cholesterol from liposomes into human red blood cells in vitro [25, 39].

Uptake by tissues

A clear result from this study was the preferential uptake of ^{14}C relative to ^{3}H from liposomes containing [^{3}H]methotrexate and [^{14}C]cholesterol. In some tissues particularly lung, this differential uptake was very high. This result together with the greater percentage of [^{3}H]methotrexate relative to ^{14}C maintained in the plasma, for sonicated liposomes suggests an independent exchange of lipids into tissue cell membranes (also discussed above). The mechanism for this will presumably involve an initial adsorption of liposomes to the plasma membrane, possibly permit-

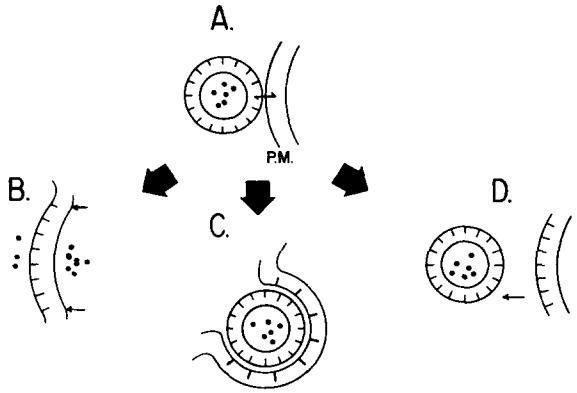

Fig. 6. Diagram of postulated modes of interaction of liposomes with plasma membranes of tissue cells. (A) Initial adsorption of liposome to cell plasma membrane (p.m.). For convenience a sonicated unilamellar liposome is shown but this would also apply to a non-sonicated multilamellar liposome. Dots indicate entrapped methotrexate and short lines indicate cholesterol and phospholipid molecules in the liposome lipid bilayer. They are arranged simply to mark the external surface and this arrangement is notintended to imply theiractual distribution. Indeed, there is evidence to suggest preferential distribution of cholesterol in the inner monolayer of unilamellar phosphatidylcholine liposomes [37]. Double arrow denotes the lipid exchange process. (B) After interaction of the liposome with the bilayer by a fusion process (see also refs. 26–28), some methotrexate is released outside the cell by this process. Small arrows indicate limits of liposome membrane and movement of lipid outside this area denotes the rapid lateral motion seen for lipids in membranes. With multilamellar liposomes presumably only the external membrane fuses and the internal membranes are released intact into the cytoplasm [26, 27]. (C) Uptake of entire liposome and contents by endocytosis. (D) Abortive adsorption possibility, suggested to explain data of present study. After exchange of lipid some liposomes are released intact.

ting a certain amount of cholesterol exchange. A number of these initial interactions, which could be termed abortive adsorptions, could result in release of the intact liposomes which retain their contents. The remaining proportion of such initial adsorptions could lead to uptake of liposomes together with part or all of their contents, either by a fusion or endocytotic mechanism as suggested by recent experiments with cultured cells [26–28]. These various possibilities are shown in diagramatic form in Fig. 6. Independent exchange of phosphatidylcholine has been detected during the interaction of liposomes with cultured cells [27, 28], but there is no evidence for independent exchange of cholesterol [28], in contrast to the exchange of cholesterol from liposomes to mitochondria and red blood cells in vitro mentioned above [24, 25, 39]. The present results suggest that such exchange processes involving liposomes also occur in vivo.

The uptake of liposomes by tissues in vivo is likely to be a highly complex and somewhat variable parameter, especially in view of the large number of possible permutations of liposome composition. Because of the expense involved in the killing of monkeys we did not do a time course of tissue uptake as other authors have done using smaller experimental animals. Monkeys, however, do have the advantage of a greater physiological and anatomical resemblance to humans for therapeutic-related studies. Also the use of larger animals such as monkeys allows the larger volumes due to the dilution produced by gel filtration of liposomes to be reliably injected intravenously. Also, it allows a complete time course of plasma levels to be

performed on the same animal, since larger plasma volumes can be obtained. The fact that values were taken at a single point for tissue uptake makes our data less precise since for certain tissues, expecially liver [3], there is an early phase of increased uptake followed by a gradual decline in tissue levels. For a number of tissues, however, the uptake is fairly constant between 1 and 6 h [29].

After correcting for plasma content it is clear, by comparing the results in Tables III–VI, that ^{3}H from liposome-entrapped [^{3}H]methotrexate is taken up considerably more than ^{3}H from free [^{3}H]methotrexate by several tissues especially liver, spleen and to a lesser degree lung. This is in agreement with a number of previous studies on other entrapped substances [1, 2, 20–22, 29], as well as [^{3}H]methotrexate [23]. Sonicating the liposomes reduces the uptake by spleen of both ^{3}H and ^{14}C quite markedly, possibly due to the fact that large liposomes, like effete red blood cells, get trapped in the spleen filtering system [30]. Also ^{3}H from [^{3}H]methotrexate in sonicated negative liposomes seems to get taken up to a much lesser extent than ^{3}H from [^{3}H]methotrexate in sonicated positive liposomes by spleen. The marked reduction in uptake for ^{3}H from [^{3}H]methotrexate and ^{3}H-labelled dipalmitoyl phosphatidylcholine in sonicated positive liposomes for a number of tissues found in our previous study at 4 h [8] was not so marked here, and may reflect an unknown degree of variability in the in vivo behavior of liposomes. Liposome entrapment also seems to partially protect against metabolic degradation of methotrexate in the tissues studied.

Potential uses of liposome-entrapped methotrexate

The therapeutic potential for liposome-entrapped substances has stimulated numerous studies both on their behavior in vivo and their interactions with cells in vitro. These studies are not comprehensively reviewed in this paper but only referred to where such work directly relates to the present study. The early suggestion of a use for liposome-entrapped enzymes in increasing the effectiveness of enzyme replacement therapy for enzyme storage diseases [3] is consistent with what appears to be their predominant localization in the lyzosomes and phagocytic vacuoles of liver cells [3, 5]. In other cases liposome-entrapped chelating agents have been reported to be useful in increasing the effectiveness of such agents in lowering the metal content of tissues after simulated heavy metal poisoning [2]. Entrapment of penicillin in negative liposomes increased the retention of this substance in the plasma [1], similar to the effect described in this paper for methotrexate, but to a lesser extent since negative liposomes are cleared more rapidly and penicillin appeared to rapidly leak out of such liposomes. However, no therapeutic effectiveness for such liposome-entrapped penicillin has been reported.

There have also been several studies on the distribution of liposome-entrapped anti-cancer drugs, and liposomes have even been detected in the tumors of a few cancer patients [31]. Actinomycin D entrapped in the lipid phase of negative liposomes has been reported to increase the mean survival time of AKR mice with AKR-A tumor cells by 50 % [6]. Liposome-entrapped actinomycin D has also recently been shown to be effective against drug-resistant cells in vitro [37]. Recent studies on the distribution of liposome-entrapped actinomycin D have shown that whereas the drug entrapped in the lipid phase showed a distribution different from that of the free drug, the distribution of actinomycin D entrapped in the aqueous phase was more

similar to that of the free drug [21]. This was attributed to the high permeability of the liposomes to actinomycin D. A similar behavior was found for $^{86}Rb^+$ or $^{22}Na^+$ entrapped in liposomes where, unlike the present results, serum caused a large increase in the in vitro permeability to these ions. Thus, only 2–3-fold differences in plasma and tissue levels were found compared to the free ions [22].

The present study shows that injection of methotrexate entrapped in liposomes provides two major potential advantages compared to injection of the free form. One, it maintains high plasma levels necessary for its therapeutic action [32], which is likely to be due in large part to inhibition of the otherwise rapid renal excretion of the free drug. It is known, however, that persistence of high plasma levels of methotrexate, which is often caused by renal impairment and a high dose regimen, leads to a general toxicity [33]. The sequestering of methotrexate inside liposomes could minimize these toxic side effects, if a degree of selective uptake of the liposome-entrapped methotrexate into tumor cells could be achieved. The second advantage demonstrated by this study is that entrapment in liposomes can prevent degradation of a drug, since methotrexate seems to be extensively metabolized in *Macaca* monkeys.

The outstanding problem for a potential chemotherapeutic use of liposome-entrapped drugs is ensuring a degree of selective uptake by the target malignant tisues with sparing of normal tissue. The possibility of altering the surface properties of liposomes to accomplish this type of specific "homing" is receiving increasing attention [34, 35]. Although some success in this regard has recently been achieved with cells in vitro by coating liposomes with immunoglobulins [34, 36], no successful specific antibodies or reports of specific "homing" in vivo have yet appeared. It would be undesirable in many cases to have a considerable uptake of cytotoxic drugs into liver and spleen cells, although the uptake by spleen can be decreased by using small sonicated, negative liposomes. If these complex problems can be solved, it is quite possible that liposome entrapment may have a general use. It is also possible that "liposomophilic" tumors may be found, against which liposome-entrapped drugs could have a more specific effect.

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