accepted model compound of a vasodilator acting through cyclic AMP phosphodiesterase inhibition (29, 30), and isoproterenol has been shown to elevate cyclic AMP levels through stimulation of adenylate cyclase. It is clear that elevated intracellular cyclic AMP levels can lead to vasodilation and that compounds that interact with either the α-adrenergic receptor or cyclic AMP phosphodiesterase may be potential vasodilators.

The presence of calcium is a necessary component of smooth muscle contraction. Several workers showed that agents that normally contract vascular smooth muscle *in vitro* cannot cause contraction when calcium is omitted from the incubation or when calcium accumulation into the tissue is blocked. Therefore, agents capable of inhibiting calcium accumulation (*e.g.* flunarizine) may act by preventing vasoconstriction and result in a net vasodilation *in vivo* (31).

It is not certain how directly the results using biochemical screening models can be extrapolated to *in vivo* conditions involving vascular smooth muscle. However, several general findings provide a basis for further experimentation and future working hypotheses.

Vasodilators must be considered a heterogeneous drug class. For example, from a therapeutic point of view, vasodilators used in the treatment of hypertension and peripheral vascular disease require different pharmacological properties. In the treatment of peripheral vascular disease, reduction of blood pressure is undesirable, whereas it is obviously a criterion for vasodilators used in hypertension (1). The optimal requirements for peripheral and cerebral vasodilators may also differ.

The actions of vasodilators clearly involve heterogeneous physiological and biochemical mechanisms related to smooth muscle contractility. It is proposed that both the therapeutic utility and side-effect pattern of a vasodilator will depend on the specific profile of the excitation–contraction coupling mechanisms altered by the drug. Additional research on this series of drugs and on future compounds will provide more specific criteria for classifying vasodilator drugs.

REFERENCES

(1) "New Antihypertensive Drugs," A. N. Richards, Ed., Spectrum, New York, N.Y., 1976, pp. 527, 533.

(2) J. Kock-Weser, *Arch. Intern. Med.*, **133,** 1017 (1974).

(3) E. Braunwald, *N. Engl. J. Med.*, **297,** 331 (1977).

(4) J. N. Cohn and J. A. Franciosa, *ibid.*, **297,** 254 (1977).

(5) "Drugs of Choice," W. Modell, Ed., C. V. Mosby, New York, N.Y., 1974, pp. 391, 398.

(6) J. D. Coffman, *N. Engl. J. Med.* **300,** 713 (1974).

(7) H. P. Bar, *Adv. Cyclic Nucleotide Res.*, **4,** 195 (1974).

(8) M. J. Berridge, *ibid.*, **6,** 1 (1975).

(9) H. Rasmassen, P. Jensen, W. Lake, N. Friedman, and D. B. P. Goodman, *ibid.*, **5,** 275 (1975).

(10) W. C. Cutting, "Handbook of Pharmacology," Appleton-Century-Crofts, New York, N.Y., 1969, pp. 201, 233, 243.

(11) J. M. Van Neuten, J. Van Beek, and P. A. J. Janssen, *Arch. Int. Pharmacodyn. Ther.*, **232,** 42 (1978).

(12) F. C. Greenslade and K. L. Newquist, *ibid.*, **233,** 270 (1978).

(13) C. Mukherjee, M. Caron, M. Coverston, and R. Lefkowitz, *J. Biol. Chem.*, **250,** 4869 (1975).

(14) L. T. Williams, D. Mullikin, and R. J. Lefkowtiz, *ibid.*, **251,** 6915 (1976).

(15) "Methods in Cyclic Nucleotide Research," M. Chasin, Ed., Dekker, New York, N.Y., 1972, pp. 69, 198.

(16) C. K. Scott, F. J. Persico, K. Carpenter, and M. Chasin, *Angiology*, **31,** 320 (1980).

(17) K. L. Newquist, F. C. Greenslade, M. Chasin, and F. J. Persico, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, **37,** 683 (1978).

(18) C. K. Scott, K. M. Krider, K. L. Newquist, M. Chasin, and F. C. Greenslade, *ibid.*, **37,** 351 (1978).

(19) F. C. Greenslade, C. K. Scott, M. Chasin, S. M. Madison, and A. J. Tobia, *Biochem. Pharmacol.*, **28,** 2409 (1979).

(20) C. A. Chidsey and T. B. Gottlieb, *Prog. Cardiovasc. Dis.*, **17,** 99 (1974).

(21) "Stimulus Secretion Coupling in the Gastrointestinal Tract," R. M. Case, and H. Boebell, Eds., Park Press, New York, N.Y., 1976, pp. 33, 47.

(22) M. E. Maguire, E. M. Ross, and A. G. Gilman, *Adv. Cyclic Nucleotide Res.*, **8,** 1 (1977).

(23) M. L. Steer, *Clin. Endocrinol. Metab.*, **6,** 577 (1977).

(24) R. Anderson, *Acta Pharmacol. Toxicol.*, **32,** 321 (1973).

(25) P. M. Lish, K. W. Dungan, and E. L. Peters, *J. Pharmacol. Exp. Ther.*, **129,** 191 (1960).

(26) R. Manhold, R. Steiner, W. Haas, and R. Kaufmann, *Naunyn-Schmiedebergs Arch. Pharmacol.*, **302,** 217 (1978).

(27) F. DeClerck, J. DeCree, J. Brugmans, and D. Wellens, *Arch. Int. Pharmacodyn. Ther.*, **230,** 321 (1977).

(28) R. J. Weed, P. L. Lacele, E. W. Merrill, G. Craib, A. Gregory, F. Karch, and F. Pickens, *J. Clin. Invest.*, **48,** 795 (1969).

(29) W. R. Kukovetz and G. Poech, *Arch. Pharmacol. Exp. Pathol.*, **267,** 189 (1979).

(30) M. S. Amer and W. E. Kreighbaum, *J. Pharm. Sci.*, **64,** 1 (1975).

(31) J. M. Van Neuten, "Mechanism of Vasodilation," Satellite Symposium, 27th International Congress in Physiological Science, Karger, Basel, Switzerland, 1978, pp. 137, 143.

Liposome Disposition *In Vivo* II: Dose Dependency

MARK E. BOSWORTH and C. ANTHONY HUNT ˣ

Received August 28, 1980, from the *School of Pharmacy, University of California, San Francisco, CA 94143.* Accepted for publication May 18, 1981.

Abstract □ The dose-dependent disposition of extruded multilamellar (diameter ~1 μm) negatively charged liposomes containing entrapped [^{14}C]inulin was studied in mice. Mice received 1500, 300, or 15 μmoles of liposomal lipid/kg iv. Carbon 14 levels were measured in the blood, liver, spleen, and carcass for 72 hr. A pronounced saturation effect, consistent with the known dose behavior of other colloids, was seen at early times; it was manifested by higher dose values in the blood and spleen but by lower liver values as the dose increased. This dose effect was attenuated in the liver but was maintained in the spleen at later times, and percent dose values approached plateau values in all tissues for all doses at later times. [^{14}C]Inulin was used as the liposomal marker because of its inability to enter cells (or, presumably, leave them if delivered there by liposomes) in its free form. An early decline in carbon 14 levels (over the first 48 hr) was seen in the liver for the low and medium doses. Because of the known ability of blood factors to cause liposomes to leak their contents, this decline was interpreted as being a loss of [^{14}C]inulin from extracellularly bound liposomes during this period. Moreover, the plateau carbon 14 levels at later times were interpreted as approximating the true level of intracellular inulin delivery by the liposomes.

Keyphrases □ Liposomes—disposition *in vivo*, dose dependency □ Disposition—of liposomes, dose dependency □ Pharmacokinetics—liposome disposition *in vivo*, dose dependency

Recently, there has been considerable interest in liposomes (phospholipid vesicles) as an *in vivo* drug delivery system. There are now ample data showing that liposome encapsulation can drastically alter the blood kinetics and tissue distribution of numerous compounds. Their potential uses in drug therapy, which were recently reviewed

0022-3549/82/0100-0100$01.00/0

(1, 2), are a direct result of these kinetic properties.

BACKGROUND

When the fate of intravenously administered liposomes is followed (*e.g., via* an entrapped aqueous space marker), the marker (and presumably the liposomes) accumulate to a significant extent in organs that contain cells of the reticuloendothelial system. This accumulation occurs principally in the liver and spleen where, depending on the liposome type, animal species, and postadministration time, up to 90% of the injected dose of the marker may be found.

The significance of this reticuloendothelial localization was noted by other investigators (3). The reticuloendothelial system is a diffuse system with cells positioned along the vasculature in the liver, spleen, and bone marrow, in the lung alveoli, and in the tissue interstices. One of its functions is to phagocytize colloidal and particulate material such as effete red blood cells, denatured proteins, cellular debris, foreign organisms, and exogenously administered substances such as colloidal carbon or gold (the term "colloid" can arbitrarily encompass suspensions with particle sizes ranging from 10 Å to several microns).

An extensive literature exists on the *in vivo* fate of these "inert" colloids (e.g., carbon and gold) (4). After intravenous administration, they are taken up primarily by reticuloendothelial cells in the liver and spleen by a two-step process, first involving binding to the cell and then phagocytosis; the latter step is presumed to take place quickly after the binding (5). In fact, this assumption is made whenever the blood disappearance rate of one of these colloids is used as a general indicator of reticuloendothelial system function (4). This dose-dependent process exhibits saturability in two respects. First, as the dose (*i.e.*, number of particles) is increased, the rate of colloid removal from the blood is decreased (6). Second, the tissue distribution changes with the dose (5). At very low, nonsaturating doses, for which blood flow to the reticuloendothelial tissues is the rate-limiting factor in the removal of the colloid from the blood, 95% or more of the colloid dose is taken up by the liver and the rest by the spleen. As the dose increases, the spleen takes up an increasingly larger fraction of the dose due to the saturation of the liver uptake. At extremely high doses, the lung and bone marrow can take up significant fractions also, in what amounts to a system-wide mobilization in response to a saturating dose (7).

Because liposomes are colloidal, and because of their well-documented reticuloendothelial system localization, it is reasonable to expect them to behave similarly to other colloids. The present study examined the possible dose dependency of liposome blood kinetics and tissue distribution. Other researchers reported limited evidence that suggests that liposome disposition may be dose dependent (8–10). The possibility of a time dependence in the dose effect was examined in the present study by following blood and tissue levels for 72 hr. An appropriate liposome marker, [^{14}C]inulin, was used to assess the extent to which the liposomes delivered their entrapped contents intracellularly.

EXPERIMENTAL

Chemicals—Purified egg yolk lecithin, sodium dipalmitoyl phosphatidate, cholesterol, and α-tocopherol (vitamin E) were chromatographic grade[1]. [Carboxy-^{14}C]inulin[2] was 2.3 μCi/mg, and scintillation reagents[3,4] were used. All other chemicals were analytical reagent grade or better. Phosphate-buffered saline (I) contained 92 m*M* sodium chloride, 23 m*M* dibasic sodium phosphate, and 11 m*M* monobasic sodium phosphate. Prior to use, phosphatidic acid was obtained by chloroform extraction of a solution of sodium phosphatidate in 0.4 *N* HCl with 20% methanol.

Preparation of Liposomes—Liposomes were prepared essentially as previously described (10) and were composed of egg lecithin, phosphatidic acid, cholesterol, and α-tocopherol in the molar ratios 4:1:1:0.05. The lipids were dissolved in chloroform, mixed in a round-bottom flask, and subsequently evaporated to dryness under vacuum. The buffer (I) containing 5 m*M* [^{14}C]inulin was added to the dried lipid to give a concentration of 37.5 μmoles of lipid/ml and then was agitated by hand (at room temperature after nitrogen sparging) until all of the lipid was suspended. This suspension was then placed in a 25-mm stirred ultrafiltration cell[5] fitted with a 1.0-μm pore size polycarbonate membrane[6] and extruded twice at a flow rate of ~10 ml/min, controlled by nitrogen pressure.

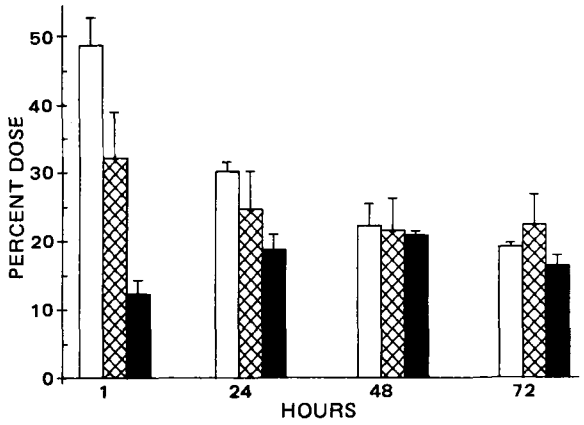

Figure 1—*Percent of injected carbon 14 dose in the liver for low (□), medium (▨), and high (■) doses at four times [mean ± 1* SD (n = *3, 10, and 4 for low, medium, and high doses, respectively)]. Equality of low and high doses was tested, and the differences between doses were statistically significant at 1 and 24 hr but not at 48 and 72 hr (one-tailed unpaired* t *test, α 0.05).*

Liposomes for the high dose were concentrated by adding 0.3 ml of 50% (w/v) sucrose to 1.1 ml of liposomes, centrifuging at 12,000 × *g* for 15 min, removing the upper concentrated liposome layer, and resuspending it in I to give a final concentration of 112.5 μmoles of lipid/ml. Liposomes were dialyzed overnight at 5° against I in dialysis cells fitted with 0.8-μm pore size membranes[6] to remove nonentrapped inulin and to improve the size distribution by removing the smallest liposomes (11). At least 10 buffer changes were done for each cell during the dialysis.

Tissue Distribution Studies—Liposomes were taken off dialysis and kept at 5° throughout the injection period. Male ICR mice, 18–24 g, were injected with 37.5, 7.5, or 0.375 μmoles of lipid/mouse (high, medium, and low doses, respectively) intravenously *via* the tail vein with 0.25–0.35-ml injection volumes. Mice receiving imperfect injections were not used. To determine the percentage of free inulin in each dose, a sample was applied to a 5 × 120-mm column of Sephadex[7] G-200-120 equilibrated and subsequently eluted with I; the eluate fractions were analyzed by scintillation counting, and the percent of inulin entrapped was calculated based on the carbon 14 recovered in the void volume and free inulin fractions.

Mice were anesthetized with ether at 1, 24, 48, or 72 hr after injection, and ~0.5–1.0 ml of blood was quickly removed from the jugular vein with a heparinized syringe. The mice were then sacrificed; the livers, spleens (and, in some instances, the brain, kidneys, and lungs) were removed and frozen for later analysis. In some cases, an aliquot of the blood was cen-

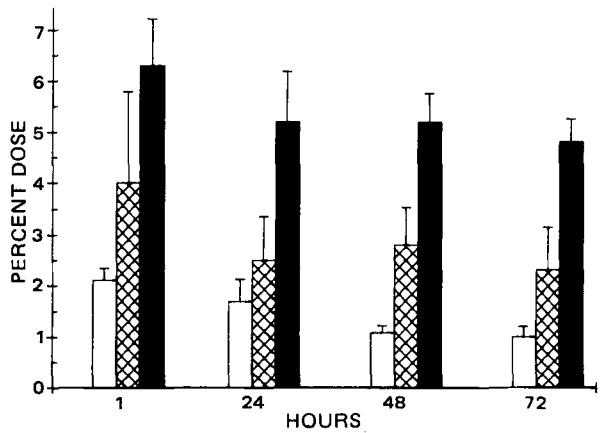

Figure 2—*Percent of injected carbon 14 dose in the spleen for low (□), medium (▨), and high (■) doses at four times [mean ± 1* SD (n = *3, 10, and 4 for low, medium, and high doses, respectively)]. Equality of low and high doses was tested, and the differences between doses were statistically significant at all time points (one-tailed unpaired t test, *α 0.05).*

[1] Sigma Chemical Co., St. Louis, Mo.
[2] New England Nuclear, Boston, Mass.
[3] Carbosorb and Permafluor V, Packard Instrument Co., Downer's Grove, Ill.
[4] PCS, Amersham, Arlington Heights, Ill.
[5] Millipore, Bedford, Mass.
[6] Nucleopore, Pleasanton, Calif.
[7] Pharmacia Fine Chemicals, Sweden.

Table I—Data [a] for Various Organs at 1 hr after Liposome Doses

Dose	Blood Levels, percent dose/ 1.0 ml (Ref.)	Percent Entrapped in Blood	Lungs	Kidneys	Brain
Low	0.39 ± 0.1 (3)	[b]	0.09	—	—
Medium	1.1 ± 0.71 (9)	10.1 ± 2.0 (3)	0.23 ± 0.04 (4)	0.825 ± 0.28 (4)	0.095, 0.18
High	10.4 ± 1.1 (4)	93, 95	0.75, 0.97	—	—

[a] Percent dose: mean ± 1 *SD* (*n*), or individual values where $n \leq 2$. [b] Blood values were too low to measure.

trifuged and a plasma sample was applied to a Sephadex column as described previously to determine the percent entrapped.

Tissue Analysis for Total Radioactivity—Duplicate aliquots of liver (0.14–0.24 g), spleen (0.04–0.08 g), whole blood (0.2 ml), and the radiolabeled liposome dose (0.05 ml) were transferred to preweighed combustion cups, which were reweighed and then allowed to air dry. The remaining carcass was placed in a container with 75 ml of water and homogenized using a wet milling device[8]. Duplicate samples of the resulting slurry (1.3–2.0 g) were weighed into combustion cups and allowed to air dry for 24 hr. Dried samples were analyzed for total carbon 14 by scintillation counting following combustion in a sample oxidizer[9].

Combustions were carried out in series, samples alternating with empty combustion cups or combustion cups containing a known amount of [^{14}C]inulin. Counts per minute were subsequently corrected for variations in quench by use of a quench curve. Results for duplicate samples were averaged, and the total radioactivity in the liver, spleen, carcass, and 1.0 ml of blood was calculated and converted to percent dose. Analysis of experimental samples and control samples of known radioactivity showed that the duplicate determinations were consistently within 5% of their mean.

***In Vivo* Clearance of Free Inulin**—In a separate study, similar doses of [^{14}C]inulin in identical volumes of I were administered to mice, which were subsequently sacrificed and analyzed as described to determine the *in vivo* clearance of free inulin.

RESULTS

Free inulin was cleared rapidly by the kidney. Its half-life in blood after intravenous injection was 10–15 min, with 0.5% of the dose remaining in blood, 2.5% in the liver, and 0.15% in the spleen at 1 hr. Carcass values at 1 hr averaged 6.9%, due presumably to residual inulin in the bladder; this value was minimized by urine removal from the bladder prior to freezing the carcass. The 24-hr value for the carcass was <1%. Thus, the presence of free inulin after the liposome doses may have contributed slightly to the 1-hr carcass data but was negligible at later times and in all other tissues.

Table I shows the 1-hr carbon 14 levels for various organs and blood, as well as the percent entrapped in blood, following the liposome doses.

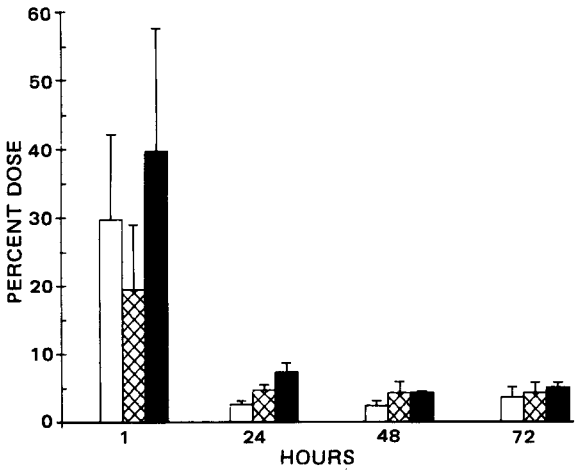

Figure 3—*Percent of injected carbon 14 dose in the carcass. Significant differences between doses were found at 24 and 48 hr but not at 1 and 72 hr (symbols and other details as in Fig. 1).*

[8] Brunwell Scientific, Rochester, N.Y.
[9] Packard Instrument Co., Downer's Grove, Ill.

Blood values, expressed as percent dose per 1.0 ml, exhibited a definite dose dependency; the values at 24, 48, and 72 hr were identical to background in all cases. Lung, brain, and kidney levels, done only in limited cases as controls, were negligible.

The other principal tissues of interest are the liver, spleen, and carcass. Figure 1 shows the results for the liver; a definite dose effect, seen as higher relative levels for the lower doses, is shown at the 1-hr time point. This effect completely diminished by 72 hr, and a similar plateau was approached by all three doses. The spleen results (Fig. 2) also showed a dose dependency but one that differed from that in the liver; the effect was maintained over the 72 hr, and higher relative levels were seen as the dose was increased. The carcass results (Fig. 3), show evidence of a dose effect at later time, which was significant at the 24- and 48-hr time points; the levels fell rapidly after 1 hr and reached a plateau. Figure 4 shows the results for the totals *in vivo*; here there is no evidence for a dose effect, and all values approach a similar plateau[10].

The reproducibility of the radioactivity measurements was also assessed. The average percent difference between duplicate samples was 6.07%, with a standard deviation of 4.23%.

DISCUSSION

The results can best be evaluated by comparison with the results one would expect for inert colloids and for liposomes (based on certain preconceived notions). Figure 5 depicts these expected dose study results. Curves A and B are plots of percent of injected dose found in all reticuloendothelial tissues *versus* time for low and high doses, respectively, of a colloid such as gold. The delayed uptake for the higher dose reflects the saturation phenomenon already mentioned. In both cases, however, all of the dose is eventually found in reticuloendothelial tissues.

The liposome case is different for two important reasons. First, it is now well known that certain factors in blood can destabilize liposomes with respect to the leaking out of their entrapped contents (13, 14). When this "leakiness" effect occurs, a significant portion of the entrapped

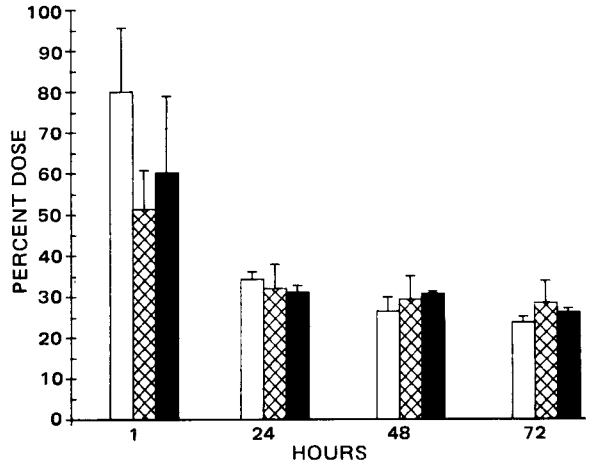

Figure 4—*Percent of injected carbon 14 dose found* in vivo *at four times. No evidence for a dose effect was seen here (symbols and other details as in Fig. 1).*

[10] In a subsequent series of studies, the mole fraction of cholesterol in the liposomes was increased to 50%, which results in liposomes with substantially increased stability in plasma (12). Following the same protocol used here and using liposome of the same size and dose, the total percent dose *in vivo* at 1 hr was 105 (*SD* ± 13%, $n = 5$); eventual plateau levels in liver and spleen were approached more slowly than in these studies.

marker can be released quickly into the blood, resulting in a <100% distribution of the marker to reticuloendothelial tissues.

The second reason is more important as far as evaluating liposome tissue distribution and concerns the nature of the entrapped marker. Most compounds that have been entrapped in liposomes have physical properties that preclude their use as accurate indicators of intracellular delivery by liposomes because these compounds are often capable of entering and leaving the target cells on their own or are metabolized to compounds that can. The resulting situation is depicted in curve E of Fig. 5, in which the tissue levels rise and fall with time due to the exit of the marker. This pattern has, in fact, been almost universally observed when tissue levels have been taken after administration of liposome-entrapped compounds.

The principal disadvantage with such data is that it is impossible to ascertain what fraction of the dose was delivered to the tissue in question. It was felt, however, that these data could be gotten using the appropriate entrapped marker. [^{14}C]Inulin was chosen because its size and polarity prevent it from entering cells on its own (or leaving, if it is delivered there *via* liposomes). It also has simple kinetics and is not metabolized. In short, it is a marker compound that should remain in the tissue to which it is delivered. The result should be tissue levels that reach plateaus at later times but, because of the leakiness effect, approach values of <100%. This situation is depicted for two different doses in curves C and D of Fig. 5.

The results of this study lead to several general conclusions. In all of the principal tissues studied (liver, spleen, carcass, totals *in vivo*) and for all doses, the levels approach plateaus at later times. This evidence supports the hypothesis that tissue level plateaus would be seen with a marker compound such as inulin; an argument will be presented later as to why these plateaus approximate the true level of intracellular delivery of the inulin by the liposomes. Furthermore, there is a pronounced dose effect at early times that is lost (in the liver) at the later times. This dose effect is consistent with that seen for other colloids such as carbon or gold; *i.e.*, the liposomes are subject to saturation kinetics, manifested by elevated relative blood and spleen levels and depressed relative liver levels as the dose increases.

As is the case for other colloids, the liver is the dominant organ in liposome disposition; because of its large blood flow and high clearance capacity relative to the other vascular reticuloendothelial tissues (*i.e.*, spleen and bone marrow), the extent to which the liver disposition of the liposomes is saturated determines the amount of colloid that is taken up by the other tissues. In other words, a delayed liver uptake results in elevated blood levels over a longer time, allowing the spleen, which is thought to have a higher cellular intrinsic activity for colloid uptake (4), to sequester a large fraction of the injected dose. The carcass values reported here (*i.e.*, everything but blood, liver, and spleen) include the bone marrow, and perhaps the slight dose effect seen in the carcass is due to uptake by the marrow.

These results also raise some intriguing questions. It is obvious that the data for the liver do not fit the predictions as depicted by curves C and D of Fig. 5. Only the high dose data fit the expected pattern of a continuous rise of the tissue levels to the eventual plateau. However, the data for the low and medium doses show a pattern with an early decline in levels followed by the plateau. Such a decline can be accounted for in only two ways.

First, a fraction of the marker that has been delivered to the cells at early times somehow partitions out of the cells at later times. However, the assumption was made earlier that the inulin marker, once delivered inside cells, would not partition out. The second explanation is that the marker levels in the liver at early times are not entirely constituted of intracellular inulin. Since the blood levels at 1 hr are very low for these two doses and since the half-life for removal of free inulin from the blood is very fast, a significant fraction of the inulin in the liver at 1 hour must be located in extracellularly bound liposomes.

It is the hypothesis of the present study that the following events occur in the liver disposition of the liposomes. If one postulates the presence of extracellularly bound liposomes, one must contend with the long-held belief that for colloids, removal from the blood can be considered the equivalent of intracellular uptake (*i.e.*, uptake occurs rapidly after binding). Data are available that show that these two phases of uptake are separable under specialized conditions *in vitro* (15), but only recently has evidence been reported that unambiguously shows that, *in vivo*, a colloid's binding to and uptake into Kuppfer cells can be very distinct events, separated by long periods of time (16, 17). In fact, Kavet and Brain (18), in their recent review on the quantification of endocytosis, emphasized that "... ingestion is not a necessary consequence of attachment. Thus, particles may become cell-associated without being interiorized and unless detected as such may be falsely included with the intracellular compartment."

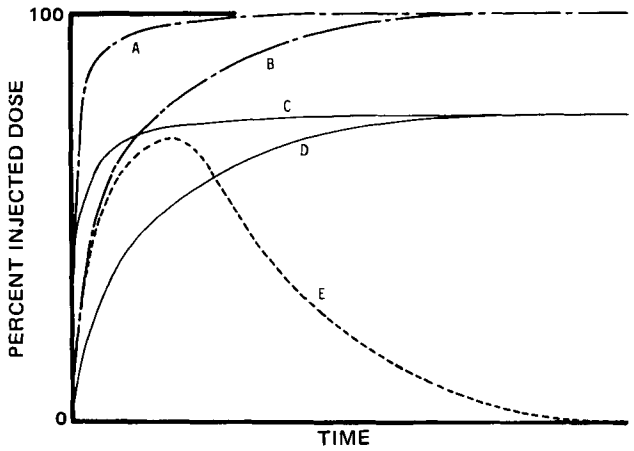

Figure 5—*Expected results for a plot of percent dose in reticuloendothelial time after intravenous administration of two different doses of colloidal gold (curves A and B), two different doses of liposome-entrapped [^{14}C]inulin (curves C and D), or a liposome-entrapped marker that can partition out of cells (curve E). These curves assume that cellular uptake of colloid occurs rapidly after binding to the tissue.*

This fact is crucial in the case of liposomes since it is very likely that extracellularly bound liposomes are just as susceptible to the destabilizing influences of blood as are free-floating liposomes. Thus, what is suggested is that: (a) tissue levels measured after administration of a liposome-entrapped marker may include substantial levels of extracellularly bound, liposome-entrapped marker, even several hours after the dose is given, and (b) these tissue levels may decline because of the slow release of marker from extracellular liposomes due to the destabilizing effect of blood.

One final piece of evidence must be considered, however, to explain completely the disposition of the three doses. Recent evidence suggests that the blood destabilizing effect may itself be saturable (13, 14); *i.e.*, as larger numbers of liposomes are incubated with a given volume of blood, a smaller fraction of the total liposome-entrapped marker is released. The liver data are thus explained as follows. In the low dose case, the liver quickly binds most of the liposome-entrapped insulin dose (part of the inulin is released from the liposomes on the initial mixing of the dose with the blood so that the initial liver value is probably <100% of the dose). However, because the intracellular uptake is slow, the bound liposomes continue to release inulin until they are taken up inside the cells. Thus, what is seen is a decline in liver levels over time until the remaining inulin is entirely intracellular and the plateau is reached[11].

In the high dose case, the initial liver binding is saturated so the relative liver levels (*i.e.*, on a percent dose basis) are low and blood levels are high. Thus, the levels of extracellular liposomes (circulating and bound) remain elevated for longer periods and are exposed to the blood's destabilizing factors longer. However, since this latter system is probably also saturated [it was calculated that the ratio of milligrams of lipid to milliliters of blood is similar to that causing saturation in *in vitro* systems (12)], the liposomes leak their contents at a relatively much slower rate. The net result is that the slowing down of the two processes of uptake (a combination of binding and endocytosis) and blood-induced liposome leakiness are offsetting effects. Thus, at later times the relative amount of inulin taken up inside cells is similar for the two doses (the medium dose is apparently an intermediate effect).

As mentioned previously, the dose behavior of the spleen conforms to the present expectations in that higher relative levels are seen as the dose increases. However, this effect, unlike that in the liver, is still evident at 72 hr. Two factors may be responsible: (*a*) the amount of colloid available to the spleen initially is largely a function of the amount bound by the liver, and (*b*) the actual intracellular uptake may be faster in the spleen so that most of what was initially bound there was also endocytosed. The carbon 14 levels in several other tissues (brain, lungs, and kidneys) were also measured (Table I). However, since the initial experiments indicated

[11] The composition of the liposomes used here was selected because of its intermediate stability in plasma (10, 14). It was conjectured that liposomes without cholesterol would rapidly lose a large fraction of their contents after injection, whereas maximally stable liposomes may result in tissue levels of carbon 14 that decline too slowly to confirm a specific final plateau level.

that very little of the dose could be found in these tissues, they were not analyzed routinely.

The liposomes used were fairly large in size (average size ~1 μm) and had a relatively narrow size distribution. This was accomplished by the use of two techniques in the sizing procedure, extrusion through 1.0-μm pore-size membranes (19) followed by dialysis against 0.8-μm membranes (11). It was felt that the excellent reproducibility seen in the tissue levels (the high dose studies were done on two separate occasions, and the medium dose study on three) was evidence of the liposome batch-to-batch reproducibility that these sizing techniques afford. The lipid composition used in the study was chosen based on *in vitro* liposome–plasma stability studies (14). The object was to choose a composition such that the liposomes were stable enough to retain most of their entrapped contents *in vivo*, yet unstable enough so that if significant long-term extracellular liposome tissue binding did take place, the marker would be released in a reasonable period (~72 hr) and thus would not be included as part of a plateau tissue level value. The decline in liver levels for the low and medium doses took place over the first 24–36 hr, which was fast enough to allow observation of the plateaus by 72 hr.

CONCLUSIONS

The results of this study have several general implications. First, it is obvious that the liposomal dose (or liposome number) is an extremely important determinant of *in vivo* disposition. It is unfortunate that *in vivo* work is still appearing in which the liposome dose is not given and is difficult or impossible to determine from the other details given [the dose in terms of micromoles of lipid/kg of body weight is currently acceptable, but the ideal would be the additional specification of liposome size and number (20)].

Second, the present data suggest that, after administration of a liposome-entrapped marker, a significant fraction of the marker may be located in extracellularly bound liposomes for long periods. Therefore, a general reinterpretation of the significance of tissue levels for most compounds administered in liposomes is needed. Specifically, tissue levels cannot be taken as an indicator of the intracellular delivery of the entrapped compound by the liposomes.

The present data suggest, however, that tissue level plateaus after administration of a liposome-entrapped compound such as inulin may be a valid indicator of intracellular delivery. This would be important for several reasons. It is necessary information for potential entrapped compounds so large or polar that their cellular uptake in the free form is zero. Moreover, for compounds that can penetrate cells to a limited extent and whose efficacy (and intracellular delivery) can benefit from a localized liposomal extracellular slow release effect, it is still important to know what fraction is delivered to cells *via* liposomes because the drug's intracellular destination may be different by different entry routes.

Finally, it is apparent that the *in vivo* system is very complex, with at least two saturable processes affecting liposome disposition and uptake by the reticuloendothelial system (which includes binding and endocytosis) and the destabilizing factors in blood. These processes compete, and their interplay determines the total organ binding and intracellular delivery of a liposome-entrapped compound. In this study, the complexity of this interplay was evidenced by the time dependence of the dose effect such that levels in the tissues approached similar relative values at later times. This finding, however, may not be true in general since the relative magnitudes of these saturable processes probably depend on (and vary at different rates with) liposome physical properties and the animal's physiological properties. These reasons make it even more important that the dose behavior of a given liposome type be well studied so that its therapeutic efficacy can be optimized.

REFERENCES

(1) H. K. Kimelberg and E. G. Mayhew, *CRC Crit. Rev. Toxicol.*, **6,** 25 (1978).

(2) D. Papahadjopoulos, *Annu. Rep. Med. Chem.*, **14,** 250 (1979).

(3) D. Papahadjopoulos, Ed., *Ann. N.Y. Acad. Sci.*, **308,** (1978).

(4) T. Saba, *Arch. Intern. Med.*, **126,** 1031 (1970).

(5) B. N. Halpern, in "Symposium on the Physiopathology of the Reticuloendothelial System," B. N. Halpern, B. Benacerraf, and J. F. Delafresnaye, Eds., 1955.

(6) G. Biozzi, B. Benacerraf, and B. N. Halpern, *Br. J. Exp. Pathol.*, **34,** 441 (1953).

(7) R. N. Baillif, *Ann. N.Y. Acad. Sci.*, **88,** 3 (1960).

(8) V. J. Caride, W. Taylor, J. A. Cramer, and A. Gottschalk, *J. Nucl. Med.*, **17,** 1067 (1976).

(9) G. Gregoriadis, D. E. Nerunjun, and R. Hunt, *Life Sci.*, **21,** 357 (1977).

(10) R. M. Abra, M. E. Bosworth, and C. A. Hunt, *Res. Commun. Chem. Pathol. Pharmacol.*, **29,** 349 (1980).

(11) M. Bosworth and C. A. Hunt, Abstracts, APhA Academy of Pharmaceutical Sciences, **9,** 94 (1979).

(12) C. A. Hunt and M. E. Bosworth, in "Drug Therapeutics: Concepts for Physicians," 1981 ed., K. C. Helman, Ed., Elsevier, North Ireland, New York, N.Y., 1981, pp. 157–174.

(13) M. C. Finkelstein and G. Weissman, *Biochim. Biophys. Acta*, **587,** 202 (1979).

(14) C. A. Hunt and S. Tsang, Abstracts, American Association for the Advancement of Science, 145th National Meeting, 1979, p. 128.

(15) M. Rabinovitch, *Exp. Cell. Res.*, **46,** 19 (1967).

(16) K. J. Donald, *Pathology*, **4,** 295 (1972).

(17) E. H. Bloch, in "Kuppfer Cells and Other Liver Sinusoidal Cells," E. Wisse and D. L. Knook, Eds., Elsevier Biomedical Press, Amsterdam, The Netherlands, 1977.

(18) R. I. Kavet and J. D. Brain, *J. Reticuloendothel. Soc.*, **27,** 201 (1980).

(19) F. Olson, C. A. Hunt, F. C. Szoka, W. J. Vail, and D. Papahadjopoulos, *Biochim. Biophys. Acta*, **557,** 9 (1979).

(20) C. Pidgeon and C. A. Hunt, *J. Pharm. Sci.*, **70,** 173 (1981).

ACKNOWLEDGMENTS

Abstracted in part from a dissertation submitted by M. E. Bosworth to the University of California in partial fulfillment of the Doctor of Philosophy degree requirements.

Supported by Health and Human Sciences Grants G-24612 and GM-26691, US Army Contract DAMD17-79C9045, the University of California at San Francisco patent fund, and a University of California Regents Fellowship.

The authors are grateful to Dr R. M. Abra and Dr. C. Pidgeon for helpful discussions and to the Chemical/Biological Information-Handling Program, National Institutes of Health, for use of the PROPHET computer system.