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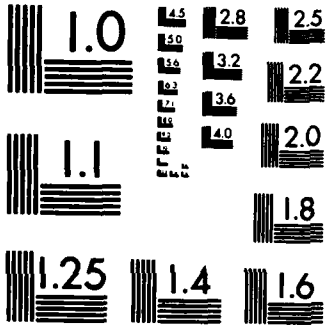

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AN INVESTIGATION OF MEMBRANE-ENCAPSULATED TRYPANOCIDES

ANNUAL REPORT

KARL J. HWANG

JANUARY 15, 1980

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

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University of Washington
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A new method which is capable of entrapping $^{111}In^{3+}$ in any types of liposomes with an efficiency of 80% ~90% has been developed. There are no differences in the physical properties and biodistribution between the liposomes encapsulating $^{111}In^{3+}$ by this new loading method and that by the conventional method of encapsulation.

Hepatic degradation of sphingomyelin:cholesterol (2/1; M/M) unilamellar

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liposomes is investigated by the combined approaches of perturbed angular correlation of gamma radiation and kinetic modeling. The hepatic degradation of the SM:CH(2/1; M/M) unilamellar liposomes in vitro at 37°C follows the first order kinetics with a half-life of 3.5 ± 0.2 hours. However, the rate of the in vivo degradiation of liposomes in the liver of a mouse is found to be 4.3 ± 0.2 hours. The rate of release of the liposome-encapsulated agent, indium-111, in the liver, is not constant and reaches a maximum at about 8 hours after the administration of liposomes.

The potential carrier application of serum protein-lipid vesicles is found to be limited. However, the SM:CH (2/1; M/M) unilamellar liposomes is found to be an excellent carrier with a blood clearance half-life of about 16.5 hours. This is by far the longest clearance time ever reported for liposomes of natural sources.

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SUMMARY

This report describes the progress of research in three specific areas, namely, the development of a method for loading a high specific radioactivity of $^{111}In^{3+}$ to liposomes, the investigation of the kinetics of the cellular degradation of liposomes *in vitro* and *in vivo*, and the search of a suitable drug carrier which can prolong therapeutic agents in blood circulation. In the first area, a new method which is capable of entrapping $^{111}In^{3+}$ in any types of liposomes with an efficiency of 80% ~90% has been developed. This method involves the transport of $^{111}In^{3+}$ to the interior of liposomes by a lipid soluble carrier, 8-hydroxyquinoline, and an efficient technique of removing $^{111}In^{3+}$ in the exterior of liposomes by an anion exchange resin. The method is very general. There are virtually no differences in the physical properties and biodistribution between the liposomes encapsulating $^{111}In^{3+}$ by this new loading method and that by the conventional method of encapsulation. This method of loading $^{111}In^{3+}$ to liposomes makes research in the investigation of the degradation of liposomes in tissues feasible.

In the second area, the approach of kinetic modeling is adopted for determining the rate of degradation of sphingomyelin:cholesterol (2/1; M/M) liposomes in livers of mice. Our results indicate that the hepatic degradation of the SM:CH (2/1; M/M) unilamellar liposomes *in vitro* at 37°C follows the first order kinetics with a half-life of 3.5 ± 0.2 hours. However, the rate of the *in vivo* degradation of liposomes in the liver is found to be slower than that *in vitro* with a half-life of 4.3 ± 0.2 hours. The rate of the release of the liposome-encapsulated agent, indium-111, in the liver is not constant, and reaches a maximum at about 8 hours after the administration of liposomes.

In the third area, the feasibility of utilizing serum protein-lipid vesicles as a potential carrier to prolong the circulation time of encapsulated pharmacological agents has been investigated. The conclusion of our study suggests that the potential carrier-application of serum protein-lipid vesicles is limited. However, the sphingomyelin:cholesterol (2/1; M/M) unilamellar liposome is found to be an excellent carrier with a blood clearance half-life of about 16.5 hours. This is by far the longest clearance time ever reported for liposomes of natural sources.

FORWARD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences - National Research Council.

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I. Loading A High Specific Radioactivity of $^{111}In^{3+}$ to Liposomes

The objective of this study is to find ways to increase the yield of encapsulating $^{111}In^{3+}$ in liposomes. This is a very important prerequisite for our subsequent investigation of the degradional process of liposomes in tissues. The yield of encapsulation of $^{111}In^{3+}$ in liposomes by standard methods of sonication is 2%~5%, depending upon the method of sonication. By using other methods of encapsulation of $^{111}In^{3+}$ in liposomes, such as the method of reverse phase evaporation, one may increase the yield of encapsulation to about 30%. However, the procedure of the reverse phase evaporation can only be used for making one special type of liposomes. Therefore, if the yield of encapsulation of $^{111}In^{3+}$ can be increased, it will not only mean a saving of materials, time and effort in preparing enough liposomes for various studies, but also make some of our future studies involving the use of liposome entrapping a high specific radioactivity of $^{111}In^{3+}$ feasible.

The method described in this progress report is a simple and general method of loading a high specific radioactivity of $^{111}In^{3+}$ to virtually any types of liposomes. The yield of encapsulation of $^{111}In^{3+}$ is generally higher than 80%. A manuscript describing the method of loading a high specific radioactivity of $^{111}In^{3+}$ to liposomes has recently been submitted for publication in Analytical Biochemistry. The results of our research in this area can be summarized in the following three paragraphs.

The principle of loading a high specific radioactivity of $^{111}In^{3+}$ to liposomes is based on the fact that the complex of $^{111}In^{3+}$ ion with 8-hydroxyquinoline (8-HOQ) is very soluble in hydrophobic environments, such as organic solvents or the bilayer of a liposome. Therefore, if the complex 8-HOQ($^{111}In^{3+}$) is added to a suspension of liposomes which entrap a water soluble chelating agent with a higher binding affinity to $^{111}In^{3+}$ than that of 8-HOQ to $^{111}In^{3+}$ the majority of indium-111 as 8-HOQ($^{111}In^{3+}$) should be first partitioned in the bilayer of liposomes and eventually become trapped in the interior of liposomes by forming complexes with the water soluble chelating agent. Since this approach of loading $^{111}In^{3+}$ to liposome depends on the partition of 8-HOQ($^{111}In^{3+}$) in the aqueous phase and in the lipid phase, a certain percentage of 8-HOQ($^{111}In^{3+}$) is expected to be in the aqueous phase outside the liposomes. A method of removing the $^{111}In^{3+}$ which is either in the external aqueous phase or on the external surface of liposomes will be required.

Based upon the above principle, we developed a method of preparing 8-HOQ($^{111}In^{3+}$) from $^{111}InCl_3$ with an efficiency of 97%-99% as shown in Figure 1. In the study of removing $^{111}In^{3+}$ ions which did not enter the interior of liposomes, it was found that the simplest method was to pass the liposomes through a small column (0.7 x 5 cm) of the anion exchange resin of AG1-X8(phosphate form) after the loading process, which involved the incubation of liposomes with 8-HOQ($^{111}In^{3+}$) at room temperature for 1 hour. Using nitrilotriacetic acid as the water soluble chelating agent entrapped in the interior of liposomes, the efficiency of encapsulation of indium-111 can be increased to 90% as shown in Figure 1. It appeared that the efficiency of loading $^{111}In^{3+}$ to liposomes depended on the amount of 8-HOQ with an optimal level of about 40 μg of 8-HOQ. Our result indicated that the efficiency of loading was not affected by the pH in the interior of liposomes over a range from pH 4.0 to pH 8.0.

The leakage of $^{111}In^{3+}$ from liposomes prepared by the present loading method and by the standard method of encapsulation was compared by measuring the release of $^{111}In^{3+}$ from liposomes incubated in either physiological salt solution or in 50% serum. Our results indicated that there was no difference between liposomes prepared by these two methods. To further study the possible difference, a comparison of the tissue distribution of liposomes prepared by both methods of encapsulation was carried out in the same animal by using dual tracers ($^{67}Ga^{3+}$ and $^{111}In^{3+}$). Gallium-67 was chosen because of its similarity to indium-111. In this case, gallium-67 was encapsulated in liposomes by the standard method of sonication and indium-111 was encapsulated in liposomes by the loading method. The mixture of these two types of liposomes was administered to a group of mice. Our results showed that the biodistribution of unilamellar dipalmitoyl phosphatidyl choline (DPPC):cholesterol (2/1, M/M) prepared by both methods of encapsulation were very similar (Table 1).

II. Degradation of Liposomes in Liver and Other Tissues

The objective of the study is to investigate the modes of cellular degradation of liposomes. The information concerning such breakdown will be extremely valuable in the application of liposomes as a means of attacking the humoral group of trypanosomes such as T. brucei, T. rhodesiense and T. gambiense or other parasites such as leishmania which grow and multiply in lysosomes of macrophages. The effort of our research can be roughly divided into two stages. In the initial stage, the emphasis of our research effort was on the characterization of the kinetics of the release of encapsulated materials from liposomes under various conditions. After selecting the suitable liposomes on the basis of their ability to resist the serum-induced leakage, the emphasis of the subsequent stage of our effort was on the determination of the rate of degradation of liposomes taken up by tissues.

(1) Characterization of the Rate of the Release of $^{111}In^{3+}$ from Liposomes. In the study of the release of entrapped $^{111}In^{3+}$ from liposomes, our results indicated that liposomes can be classified into four different categories in terms of the kinetics of the release of $^{111}In^{3+}$. Liposomes in the first category have a very high leakage rate, such that $^{111}In^{3+}$ may release from the liposomes in a matter of several hours at room temperature even if the liposomes were incubated in the absence of serum. Egg phosphatidyl choline liposomes containing various concentrations of cholesterol belong to this category.

Liposomes in the second category had a negligible rate of the release of $^{111}In^{3+}$ when incubating in physiological salt solution. However, the leakage of $^{111}In^{3+}$ was greatly enhanced by the presence of serum at room temperature. Dimyristyl phosphatidylcholine (DMPC) liposomes containing various concentrations of cholesterol up to 33% by weight belong to this category.

The third type of liposomes had a negligible rate of release of $^{111}In^{3+}$ in the presence of serum at room temperature, but they had a considerable rate of release at 37°C. DPPC:cholesterol (2/1; M/M) liposomes belong to this category. It was also found that the presence of 8% molar fraction of stearylamine or dicetylphosphate in DPPC: cholesterol (2/1; M/M) liposomes increased the leakage rate of $^{111}In^{3+}$.

On the other hand, the release of $^{111}In^{3+}$ from the fourth type of liposomes was undetectable in the presence of serum at 37°C at room temperature. Distearoylphosphatidyl choline (DSPC):cholesterol (2/1; M/M) and bovine brain sphingomyelin (SM):cholesterol (2/1; M/M) liposomes belong to this category. Our results showed that the presence of 10% molar fraction of dipalmitoyl phosphatidyl ethanolamine in the SM:cholesterol (2/1; M/M) liposomes had a negligible effect on leakage of $^{111}In^{3+}$. In the study of the release of $^{111}In^{3+}$ from this type of liposomes in vivo, our results indicated that virtually all the liposomes in this category remained intact in the circulation of mice even at 24 hours after the administration of liposomes.

(2) Kinetics of Hepatic Uptake and Degradation of Unilamellar Sphingomyelin-Cholesterol Liposomes. After the above screening process, liposomes in the fourth category were chosen as candidates for further study of their degradation in tissues. The main reason for choosing this category of liposomes is that the probability of employing them to deliver a known and controllable dose of trypanocides for chemotherapy of trypanosomes will be better than that of liposomes which release their encapsulated content. Recent study on the interaction of liposomes with the non-pathogenic trypanosome Crithidia fasciculata indicated that the uptake of liposome-encapsulated drug by the trypanosome was more than 15-fold greater than the uptake of the free drug (1). This may suggest that even in treatment of haematic group of trypanosomes, liposome-encapsulated trypanocides may be more effective than the free trypanocides.

The goal of our study on the cellular degradation of liposomes involves the investigation of liposomes of various sizes, physical properties, and chemical compositions of lipids. In view of the importance of understanding the kinetics of the degradation of first layer of liposomes, we chose SM:CH (2/1; M/M) unilamellar vesicles as the first candidate for our study. The reasons for our choice of SM:CH unilamellar liposomes are:

(i) The leakage of $^{111}In^{3+}$ from liposomes due to the mechanism of passive diffusion of $^{111}In^{3+}$ is eliminated. This will enable us to simplify our model-analysis discussed in this Section.

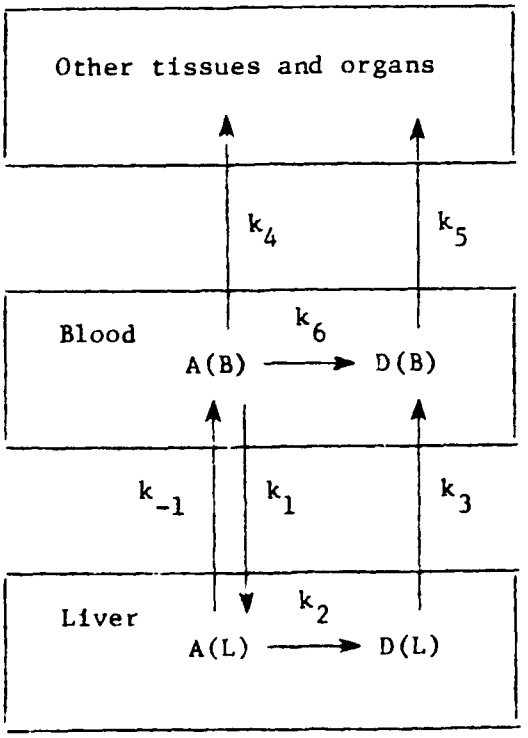
(ii) The uniform distribution of the size of SM:CH unilamellar liposomes will simplify the complexity of different rates of degradation due to different sizes of liposomes.

(iii) The long half-life time of these SM:CH unilamellar liposomes in circulation serves a convenient internal standard for checking the structural integrity of liposomes before they are degraded in liver.

Our results indicated that the rate of degradation of liposomes by the liver of a mouse was temperature dependent. The rate of degradation of liposomes was very slow at 1°C and increased with increasing temperature. The time course of the degradation of liposomes at 24°C and at 37°C is shown in Figure 2. In the control experiment, liposomes remained intact in the blood at these two temperatures. The mechanism of this temperature effect on the rate of the degradation of liposomes is not known. This could be due to the temperature effect on the activities of lysosomal enzymes, or on the process of phagosome-lysosome fusion, or on the internalization of liposomes to cells of liver. Further work will be needed to investigate the control mechanism of such degradational process.

Based on the assumption of the first order kinetics, the half-life of the in vitro hepatic degradation of SM:CH liposomes at 37°C was estimated to be 3.5 $\pm$ 0.2 hours. The comparison of the observed rate of degradation with the predicted values is shown in Figure 2. It appears that the least-squares fit between the experimental data and the theoretically predicted values is quite well.

The data of the in vivo degradation of SM:CH liposomes in liver were obtained by measuring the percentages of intact liposomes remaining in the livers from a group of mice by sacrificing them at different periods after the intravenous administration of liposomes. The rate of hepatic degradation of liposomes was analyzed by least-squares fitting the experimental data of the uptake and degradation of liposomes in various organs with a kinetic model. For simplicity, the body was subdivided into three major compartments, blood, liver and other organs.



The basic assumption of the analysis was that all rate processes involved were first order. The excretion was not considered in the present model since the renal and fecal excretion of radioactivity was negligible (about 0.5% of the injected dose in a twenty-three hour period). The amount of intact liposomes expressed as the percentage of administered dose in the blood and in the liver was denoted by A(B) and A(L), respectively. The amount of radioactivity released from the degraded liposomes to the circulating blood and to the liver was represented by D(B) and D(L), respectively. The helpatic uptake of intact liposomes from the blood was described by a rate constant k_1, while intact liposomes in the liver may return to the blood stream with a rate constant of k_{-1}. Radioactivity released from the degraded liposomes in the liver may return to the circulating blood with a rate constant of k_3. The uptake of intact liposomes and the uptake of the radioactivity released from degraded liposomes by other tissues and organs were denoted by rate constants of k_4 and k_5, respectively. The degradation of liposomes in the liver and in the blood were designated by rate constants of k_2 and k_6, respectively.

Based upon the model, the rate equations for the four observed parameters of the radioactivity of intact and degraded liposomes in the circulating blood and liver were shown below.

$$dA(B)/dt = -k_1 \times A(B) + k_{-1} \times A(L) - k_4 \times A(B) - k_6 \times A(B)$$
$$dA(L)/dt = k_1 \times A(B) - k_{-1} \times A(L) - k_2 \times A(L)$$
$$dD(L)/dt = k_2 \times A(L) - k_3 \times D(L)$$
$$dD(B)/dk = k_3 \times D(L) - k_5 \times D(B) + k_6 \times A(B)$$

The solutions at any time, t, were obtained by the least-squares analysis using the IMSL (International Mathematical and Statistical Library) subroutine, DVERK, which utilizes a Runge-Kutta method based on Vernier fifth and sixth order pair of formula (2). From these solutions, the total amount of radioactivity in the blood, the total amount of radioactivity in the liver, and the percentage of intact liposomes remained in the blood and the liver were calculated.

From the above analysis, the rate constant of the hepatic degradation of SM:CH unilamellar liposomes and other rate constants involved in the uptake and degradation of liposomes in the above model were calculated and shown in Table 2. Using these rate constants, it was possible to predict the total amount of radioactivity from intact and degraded liposomes present in the blood (Figure 3) and in the liver (Figure 4), and the percentage of intact liposomes remained in the liver (Figure 5). Good agreement between the predicted values and the experimental observations is apparent. It appears that the *in vivo* degradation of liposomes in the liver obeys the first-order kinetics as well. The half-life of the *in vivo* hepatic degradation of liposomes was found to be 4.3 ± 0.2 hours, slightly longer than the results obtained from *in vitro* measurements of an isolated liver. The instantaneous hepatic degradation rate of liposomes at various times post-administration (Figure 6) can also be calculated from the rate constants in Table 2. This is an extremely important parameter since it is related to the amount of the encapsulated agent available in the liver for pharmacological action at any time post-injection. Our results indicated that the degradation rate of SM:CH small unilamellar liposomes is maximal at 8 hours post-injection.

The approach of using a mathematical model to fit the experimental data can be applied to study liposome degradation in any organs or tissues. The apparent good fit between the experimental data and the theoretically calculated values suggests that our pre nt model is probably sufficient to describe the dynamics of the in vivo degradation of liposomes in mouse liver. Conceivably, the approach described above will not only be useful for determining the rate of the degradation of liposomes with various physical properties and chemical compositions, but also be useful for investigating factors which may control the release of pharmacologically active agents in tissues. A manuscript describing the above approach of the analysis and the results of the analysis has recently been accepted for publication in Proc. Natl. Acad. Sic. USA for publication.

There are several things that are worth pointing out. The model that we used was not completely perfect. Although the present least-squares fit was quite good, the degradation in other tissues and the excretion process were not included in the model. Work is in progress to include these processes and to extend our analysis of the data beyond 24 hours. Furthermore, we noticed that the dependence of the uptake and degradation of liposomes on the concentration of liposomes could be important. This may be one of the reasons to explain the difference between the in vitro and in vivo rates of hepatic degradation of SM:CH liposomes. Further studies will be required to investigate the effect of the concentration of liposomes on the uptake and degradation.

III. Prolongation of Drug Carrier in Blood Circulation

The objectives of this study are to explore the possibility of utilizing serum protein-lipid vesicles as a means to prolong the encapsulated materials in blood circulation and to search other means to control the blood clearance time of drug carriers. The results of our study are summarized in the following two separate sections.

(1) Characterization of Serum Protein-Lipid Vesicles. Our research effort was directed toward the study of lipid-protein vesicles made from sera of rats and rabbits. Several different types of markers were used for the encapsulation experiments. These include $^{99m}TcO_4^-$, complexes of $^{111}In^{3+}$ to ethylenediamine-tetraacetic acid (EDTA) or trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA), and phosphate. In all the cases, we found that the amount of markers associated with serum protein-lipid vesicles after sonicating serum and markers together were several times more than that after incubating markers with serum protein-lipid vesicles which were prepared by sonication prior to the incubation. However, the yield of encapsulation was very small in all the studies. They were generally less than 0.5% for both the sera of rats and rabbits.

In investigating the difference between rabbit serum and rat serum, we found that the efficiency of encapsulation for rabbit serum was higher than that for rabbit serum. However, the same trend of a higher nonspecific binding of markers to rabbit serum protein-lipid vesicles was also observed. Rat serum was thus chosen for our further investigation.

Our subsequent effort was on characterizing the nature of the encapsulation or association of radioactive markers with the serum protein-lipid vesicles. Our primary concern was on the amount of markers bound to serum protein-lipid vesicles vs. the degree of the true encapsulation of the radioactive markers by serum protein-lipid vesicles. In doing this, a number of markers were tested for their ability to bind to serum proteins. Two radioactive markers with negligible affinity to serum proteins were chosen for further characterization. These two markers were the complex of $^{111}In^{3+}$ with CDTA and $^{99m}TcO_4^-$ which was the radioactive marker reported in the original literature about the preparation of serum protein-lipid vesicles.

Several approaches were used to examine the release of the radioactive marker from the serum protein-lipid vesicles. These included(1) the dialysis of the serum protein-lipid vesicles in the presence of Triton X-100, and (2) the separation protein bound radioactive markers from free markers in a Sephadex G-50 column after incubating the serum protein-lipid vesicles with Triton X-100 or after extracting the lipid from serum protein-lipid vesicles by isopropyl ether:butanol (60:40). In all instances, we found that more than 80% of the radioactive markers appeared to be protein-bound. This suggested that the radioactive markers associated with serum protein-lipid vesicles were not entrapped by the lipid bilayer of vesicles.

Our next study was to investigate how the addition of synthetic lipids to serum may change the formation of serum protein-lipid vesicles. Using bovine brain sphingomyelin and cholesterol as the supplemented lipids to rat serum, we found that the percentage of markers associated with vesicles can be increased as we originally anticipated. However, the amount of markers bound to serum proteins appeared to be not affected by the presence of exogenous lipids. Our animal studies on the blood clearance of serum protein-lipid vesicles prepared in the presence of sphingomyelin:cholesterol (2/1; M/M) was quite similar to the vesicles prepared from the sphingomyelin and cholesterol. In fact, we found that the spleenic uptake of serum protein-lipid vesicles supplemented by sphingomyelin and cholesterol was higher than that of the synthetic lipids alone.

The conclusion of our study suggests that the potential carrier-application of serum protein-lipid in vesicles is limited. The reasons are (1) the apparent low yield of association of markers with protein-lipid vesicles; (2) the lack of evidence of entrapment of markers by the lipid bilayer of the protein-lipid vesicles; (3) the inability to improve the blood circulating time of liposomes.

(2) Long Blood Clearance Time of Sphingomyelin Liposomes. In the process of characterizing the properties of various types of liposomes, we made a very interesting and promising discovery in the biodistribution of sphingomyelin:cholesterol (2/1; M/M) liposomes. After intravenous injection of the SM:CH unilamellar liposomes to mice, the distribution of the liposome-encapsulated radioactivity in 14 tissues at four representative time points is shown in Table 3. The $\langle G_{22}(\infty) \rangle$ value of the blood samples were 0.59 ± 0.02 regardless of the time that the mice were sacrificed. This indicated that all the

radioactivity observed in the blood samples were from intact liposomes. It was noted that about 30% of the administered liposomes were cleared from the blood within 15 minutes post-adminstration of liposomes. However, more than 50% of administered liposomes still remained in the circulation at 12 hours post-injection. As calculated from the data in Figure 3 by least-squares fitting, the elimination half-life of SM:CH liposomes was found to be 16.5 hr., which is by far the longest clearance time ever reported for liposomes of natural sources.

As can be seen in Table 2, there was a rapid uptake of the liposomes by various tissues within 15 min. of the adminstration of liposomes. Liver was not the major organ responsible for such uptake. The nature of this rapid uptake is not known. Further study will be needed to investigate whether this uptake represents the amount of liposomes leaving the vascular system or the liposomes taken up by the tissues. The ability for the SM:CH liposomes to distribute in various tissues and to stay in blood circulation for an extended period of time make it an ideal candidate for further investigation in carrying trypanocides for treatment trypanosomes. Moreover, the SM:CH liposomes may also be an ideal system for investigating the approach of targeting *in vivo*.

REFERENCES

1. Vakirtzi-Lemonias, C. and Gregoriadis, G.: Uptake of liposome-entrapped agents by trypanosome Crithidia fasciculata. Trans. Biochem. Soc. 6: 1241-1244, 1978.

2. Hull, T.E., Enright, W.H. and Jackson, K.R. User's Guide for DVERK - a Subroutine for Solving Non-stiff ODE's, TR No. 100, Department of Computer Science, University of Toronto, 1976.

TABLE 1. Biodistribution of Small Unilamellar DPPC:cholesterol (2/1; M/M) Liposomes Encapsulating $^{67}Ga^{3+}$ by Sonification and $^{111}In^{3+}$ by 8-HOQ ($^{111}In^{3+}$) Loading*

Tissue	Percentage of Administered Dose at Various Times Post-Injection							
	1 hr.		3 hr.		5 hr.		23 hr.	
	In-111	Ga-67	In-111	Ga-67	In-111	Ga-67	In-111	Ga-67
Blood	47.8(4.3)	49.5(10.6)	42.9(4.3)	44.8(9.1)	36.0(1.7)	39.6(5.0)	8.4(2.1)	13.5(4.9)
Liver	8.7(1.9)	6.8(0.6)	11.5(1.5)	10.0(1.0)	15.0(1.9)	12.8(1.0)	30.3(1.6)	26.2(1.1)
Kidney	4.5(2.0)	1.1(0.0)	4.5(1.0)	1.3(0.3)	4.5(0.7)	1.4(0.2)	6.6(0.8)	2.6(0.4)
Spleen	0.6(0.3)	0.5(0.1)	1.0(0.1)	0.9(0.1)	1.4(0.1)	1.3(0.3)	2.2(0.3)	2.1(0.6)
Heart	0.6(0.4)	0.5(0.4)	0.3(0.1)	0.3(0.2)	0.4(0.1)	0.3(0.2)	0.4(0.1)	0.3(0.1)
Lung	1.6(2.0)	1.3(1.7)	1.2(1.3)	1.1(1.2)	0.7(0.1)	0.6(0.1)	1.0(0.4)	1.0(0.5)
Intestine	2.5(0.3)	3.8(1.1)	4.4(0.9)	6.6(2.0)	5.4(0.2)	7.1(0.8)	9.6(0.7)	12.6(1.3)
Fat	1.4(0.8)	1.4(0.9)	1.4(0.6)	1.5(0.6)	1.6(0.7)	1.5(0.8)	1.6(0.6)	1.4(0.8)
Skin	9.3(2.3)	9.8(2.9)	9.3(1.9)	9.5(2.8)	11.1(2.6)	10.2(3.2)	11.4(2.0)	8.3(1.6)
Tail	2.4(1.7)	2.5(1.8)	2.4(1.2)	2.4(1.2)	2.2(1.9)	2.2(1.7)	1.9(0.1)	2.1(0.1)
Legs	4.6(2.1)	5.2(3.3)	5.1(1.0)	5.2(1.4)	5.3(1.2)	5.5(1.5)	6.9(1.1)	7.5(1.2)
Carcass	15.4(2.4)	16.8(4.3)	15.4(0.8)	15.7(2.6)	16.0(1.3)	16.8(2.5)	18.7(1.7)	21.2(4.1)
Brain	0.2(0.1)	0.2(0.1)	0.1(0.0)	0.1(0.0)	0.1(0.1)	0.1(0.1)	0.1(0.0)	0.1(0.0)
Stomach	0.4(0.1)	0.5(0.2)	0.4(0.0)	0.5(0.0)	0.4(0.1)	0.5(0.2)	0.8(0.3)	1.0(0.4)

*The data represent an average of four independent studies. The number in the parenthesis is the average deviation from the mean.

Table 2. Calculated rate constants of the uptake and degradation of SM:CH unilamellar liposomes

Rate Constant	*In vitro* (a) (1/hr)	*In vivo* (a) (1/hr)
k_1	0.0	0.022 ± 0.002
k_{-1}	0.0	0.010 ± 0.010
k_2	0.200 ± 0.010	0.165 ± 0.015
k_3	0.0	0.0 ± 0.001
k_4	0.0	0.020 ± 0.005
k_5	0.0	0.0 ± 0.001

(a) Average of two independent determinations

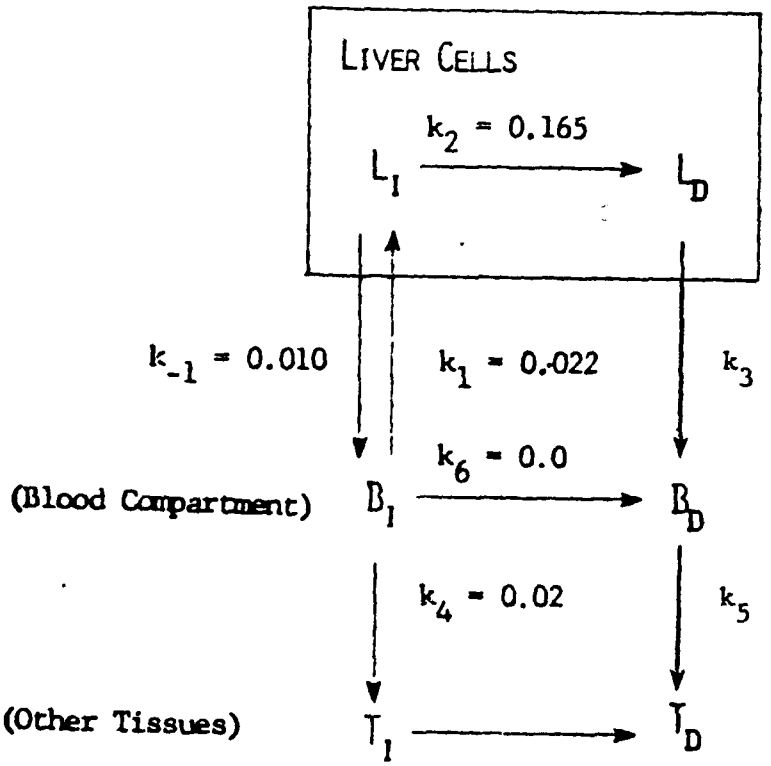


Table 3. [illegible] of the Biodistribution of Injected Liposomal Radioactivity in [illegible].

Tissue	Percentage of Administered Dose at Various Times Post-Injection n = 4 0.25 hr.	n = 4 3.0 hr.	n = 2 12.0 hr.	n = 3 23.0 hr.
Blood	71.5 ± 7.0	65.6 ± 1.1	50.9 ± 7.0	28.4 ± 7.5
Liver	5.3 ± 2.2	6.6 ± 0.2	14.7 ± 1.1	27.5 ± 6.8
Kidney	1.1 ± 0.1	1.6 ± 0.4	1.5 ± 0.3	2.2 ± 0.3
Spleen	0.2 ± 0.1	0.3 ± 0.1	1.1 ± 0.4	1.9 ± 0.2
Heart	0.9 ± 0.3	0.9 ± 0.2	0.7 ± 0.2	0.7 ± 0.2
Lung	2.9 ± 0.9	2.1 ± 0.5	1.1 ± 0.3	1.2 ± 0.4
Stomach	0.4 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	0.5 ± 0.1
Intestine	3.0 ± 0.7	3.9 ± 0.4	4.2 ± 0.2	6.8 ± 1.1
Fat	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	1.0 ± 0.1
Tail	2.5 ± 0.5	1.8 ± 0.6	1.6 ± 0.5	2.6 ± 0.6
Skin	3.6 ± 0.7	4.3 ± 0.3	6.2 ± 2.0	8.3 ± 3.1
Brain	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
Legs	2.3 ± 0.5	2.8 ± 0.3	3.3 ± 0.3	4.7 ± 1.5
Carcass	11.8 ± 2.1	10.0 ± 1.0	10.4 ± 0.4	12.1 ± 2.0

***The errors listed are the maximal deviation from the mean.**

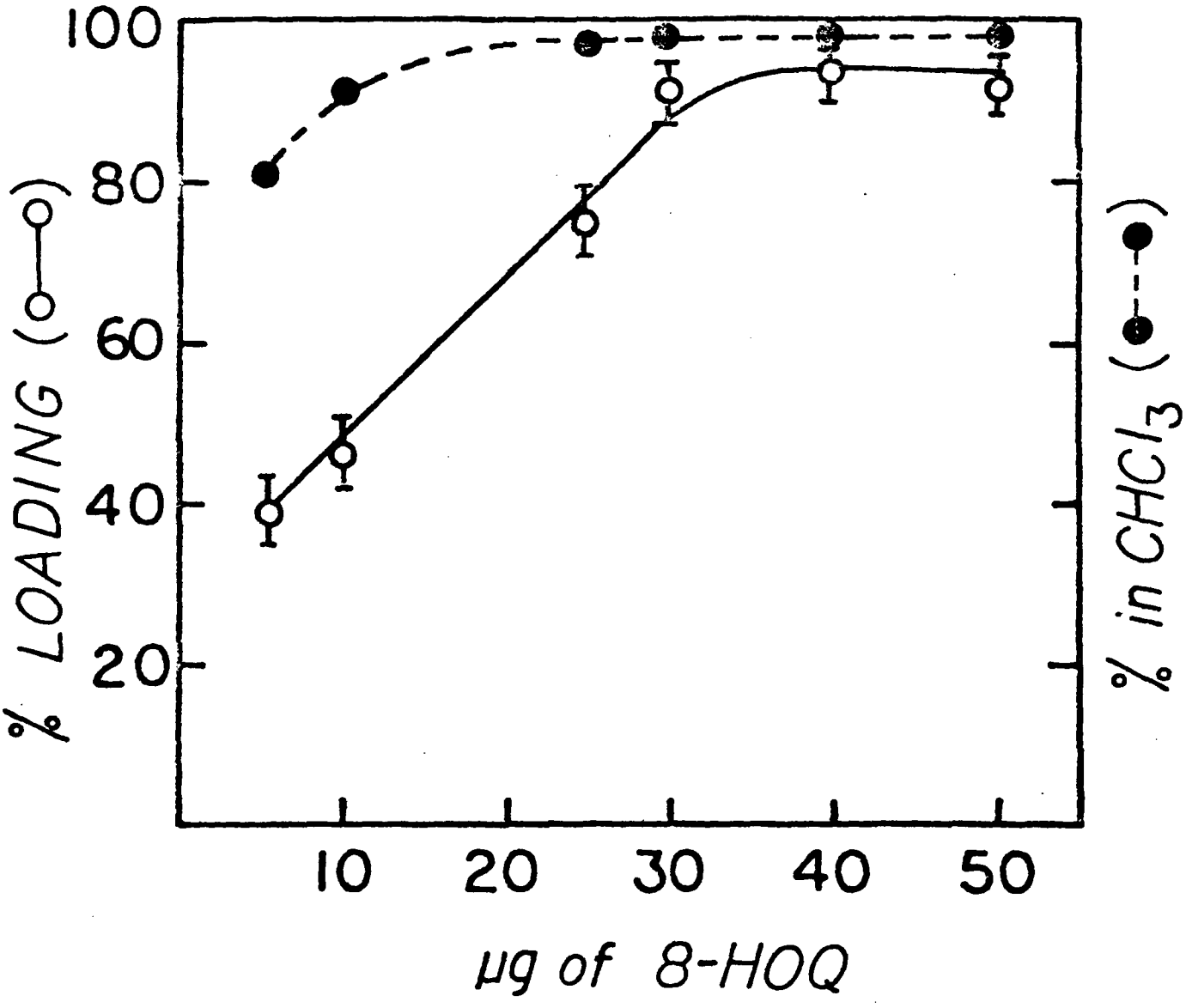


FIGURE 1. Efficiency of extracting 8-HOQ($^{111}In^{3+}$) to chloroform and loading $^{111}In^{3+}$ to DPPC:CH(2/1;M/M) liposomes. 8-HOQ($^{111}In^{3+}$) was prepared by incubating $^{111}InCl_3$ with 270 μl solution of 200mM sodium acetate (pH 5.5), 18% ethanol containing various amounts of 8-HOQ for 10 min. at room temperature prior to the extraction of 8-HOQ($^{111}In^{3+}$) by chloroform (●—●). The extracted 8-HOQ($^{111}In^{3+}$) was dried at 85°C to remove the chlorom and dissolved in 25 μl∿50μl ethanol. The loading of $^{111}In^{3+}$ to DPPC:CH liposomes entrapping 1mM NTA was carried out by squirting the 8-HOQ($^{111}In^{3+}$) in ethanol to 0.5 ml of liposomes in a glass test tube while the solution was mixed by gentle vortexing. The solution was incubated at room temperature for one hour. The yield of loading $^{111}In^{3+}$ to liposomes (o-o) was determined by counting the radioactivity in liposomes and in the resin of AG1X8 after the procedure of removing $^{111}In^{3+}$ in the exterior of liposomes by a small column (0.5X7 cm) of AG1X8 (phosphate form).

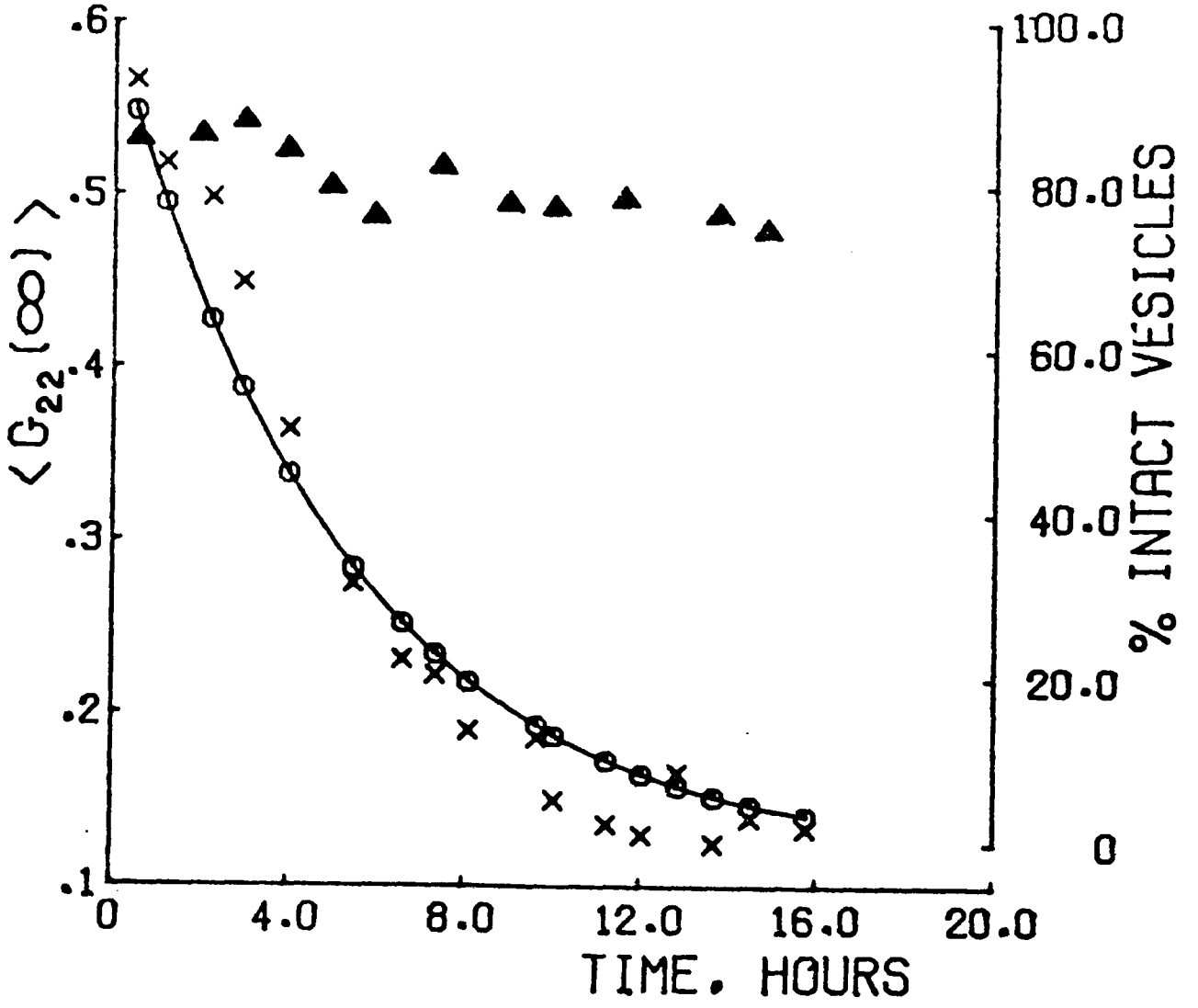


FIGURE 2. In vitro degradation of SM:CH liposomes in an excised liver at two different temperatures. The liver was removed from a mouse sacrificed at 15 minutes post-administration of liposomes. The percentage of intact liposomes in the liver was determined from the measurement of the time integrated perturbation factor, $\langle G_{22}(\infty) \rangle$, which is the parameter measured by the technique of gamma-ray perturbed angular correlation. The $\langle G_{22}(\infty) \rangle$ values of the liver sample were measured at 37°C(X) and 24°C(▲). The predicted $\langle G_{22}(\infty) \rangle$ values (o-o) were calculated, assuming that the degradation of liposomes in liver follows the first order kinetics. The percentage of intact liposomes corresponding to the $\langle G_{22}(\infty) \rangle$ values shown on the right axis was estimated by the following equation:

$$\langle G_{22}(\infty) \rangle_t = X_t \langle G_{22}(\infty) \rangle_{intact} + (1-X_t) \langle G_{22}(\infty) \rangle_{degraded} \quad (1)$$

where X_t represents the percentage of intact vesicles in the liver at the time, t, post-injection. The values of $\langle G_{22}(\infty) \rangle_{intact} = 0.59$ and $\langle G_{22}(\infty) \rangle_{degraded} = 0.12$ were used for intact and completely disrupted liposomes, respectively.

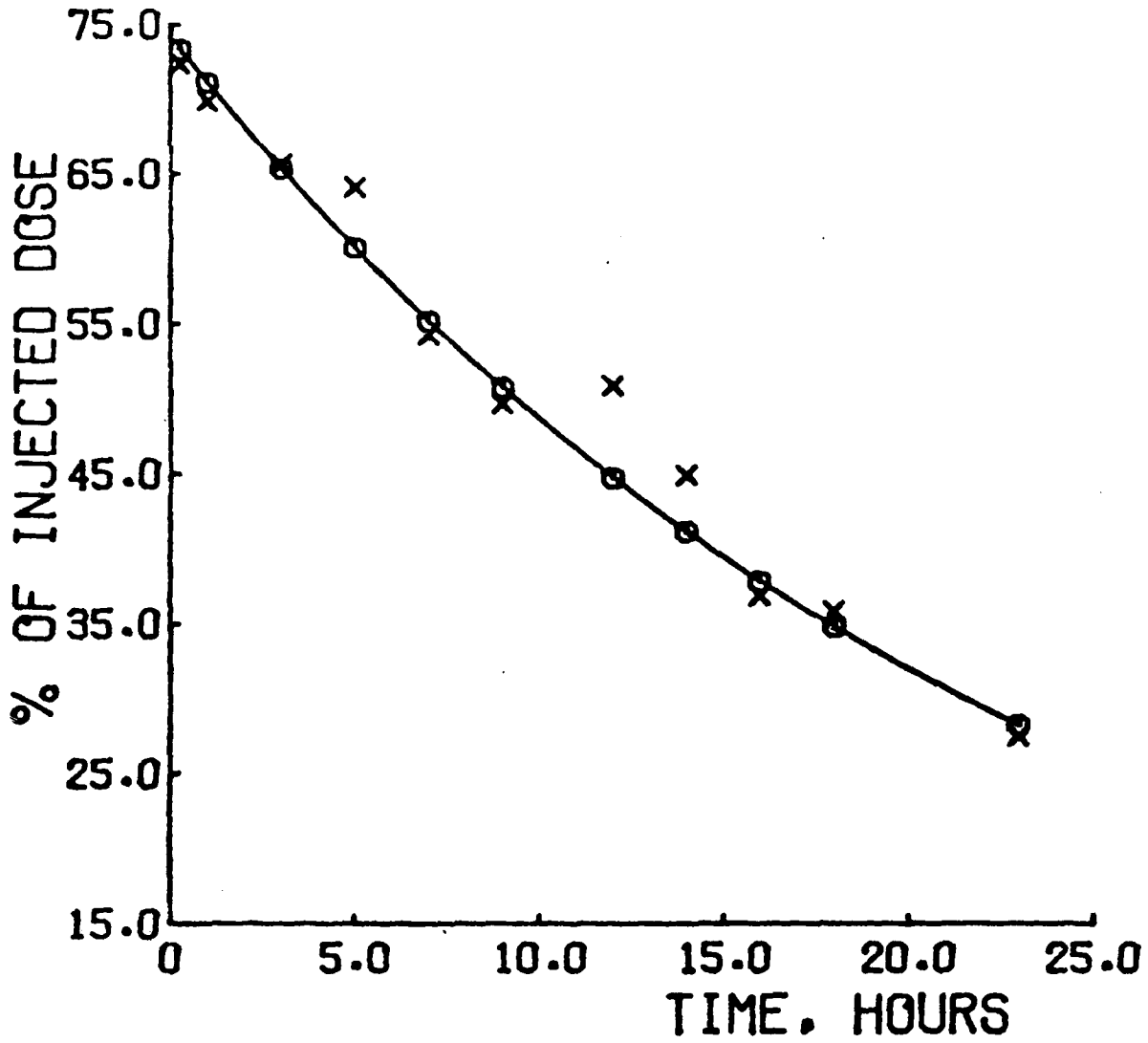


FIGURE 3. The clearance of the total radioactivity of intact and degraded liposomes from the blood. The experimental data (X) are compared with the predicted values (O-O) calculated from the rate constants listed in Table 2.

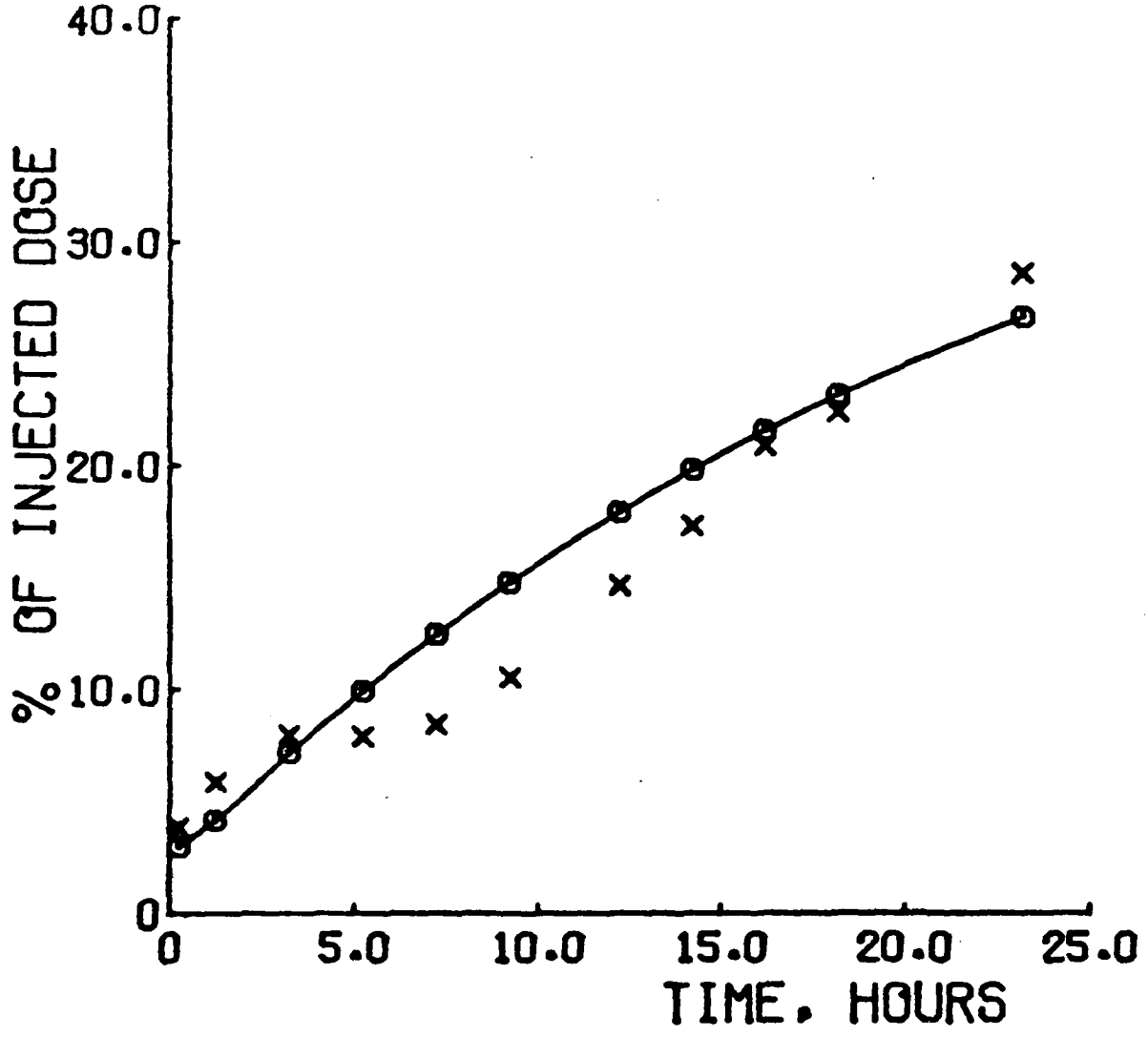


FIGURE 4. The accumulation of the total radioactivity of intact and degraded liposomes in the liver. The experimental data (X) are compared with the predicted values (0-0) calculated from the rate constants listed in Table 2.

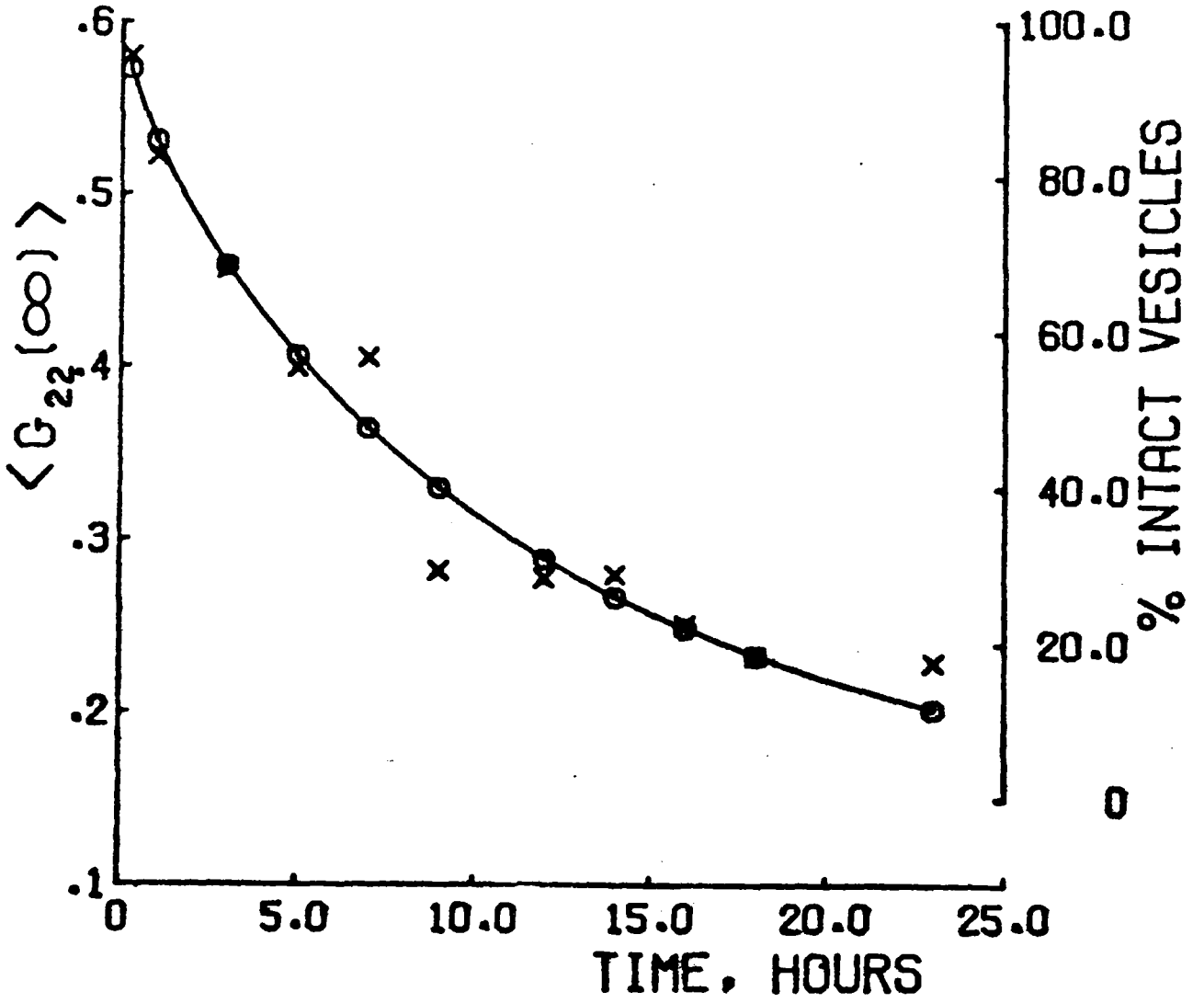


FIGURE 5. The observed and predicted percentages of intact liposomes in the livers of mice. The observed $\langle G_{22}(\infty) \rangle$ values (X) were determined as described in the legend of Figure 2. The predicted $\langle G_{22}(\infty) \rangle$ values (0-0) were calculated from equation (1) and the solutions of the rate equations using the rate constants listed in Table 2. The percentage of intact liposomes corresponding to the $\langle G_{22}(\infty) \rangle$ values shown on the right axis was calculated as described in the legend of Figure 2.

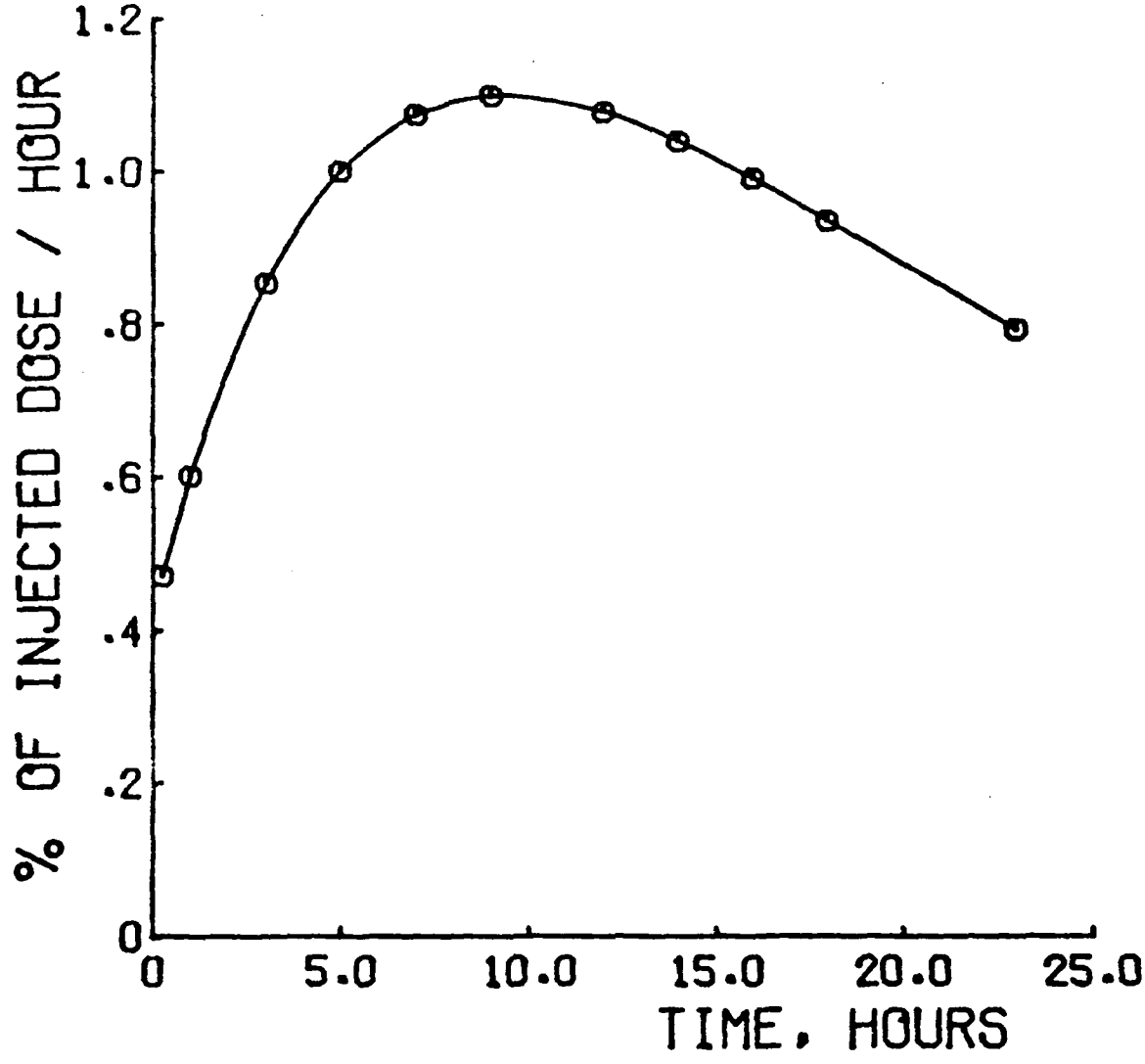


FIGURE 6. The predicted instantaneous degradation rate of SM:CH (2:1) liposomes in the liver of a Balb/c mouse at various time after the administration of liposomes. The calculation was based on the rate constants listed in Table 2.

GLOSSARY

8-HOQ:	8-hydroxyguinoline
8-HOQ($^{111}In^{3+}$):	the complex of 8-HOQ with $^{111}In^{3+}$
DPPC:	L-α-dipalmitoyl phosphatidylcholine
DSPC:	L-α-distearoly phosphatidylcholine
SM:	bovine brain sphingomyelin
CH:	cholesterol
PAC:	perturbed angular correlation
$<G_{22}(\infty)>$:	time integrated perturbation factor. This is the parameter measured by the counters of gamma-ray perturbed angular correlation spectrometer. The value of $<G_{22}(\infty)>$ has a range from 0.0 to 1.0. In the system of liposomes, a low value of $<G_{22}(\infty)>$ closed to 0.0 means that the liposomes are greatly perturbed and release their encapsulated contents. On the other hand, intact liposomes has a characteristic high $<G_{22}(\infty)>$ of about 0.60.
EDTA:	ethylenediamine-N,N,N',N'-tetraacetic acid
CDTA:	trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid

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