Prolonged Hypoglycemic Effect in Diabetic Dogs Due to Subcutaneous Administration of Insulin in Liposomes

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SUMMARY

The biologic action of insulin entrapped in liposomes (phospholipid vesicles) has been investigated following subcutaneous injection to dogs made diabetic with a combination of alloxan and streptozotocin. The fate of the liposomally entrapped material was determined by injecting rats subcutaneously with either ^{125}I-insulin or the labeled polysaccharide ^{14}C-inulin, incorporated in liposomes labeled with ^{3}H-cholesterol.

Injection of liposome insulin (0.75 U/kg) to five diabetic dogs resulted in a mean (± SEM) blood glucose fall from 16.4 ± 0.8 to 2.9 ± 0.4 mmol/L. The glucose level had still not returned to baseline after 24 h and, correspondingly, immunoreactive insulin (IRI) could still be detected in frozen and thawed plasma 24 h after injection. In contrast, the hypoglycemic effect of the same dose of free insulin with or without empty liposomes virtually ended within 8 h and IRI levels returned to baseline by 3 h after injection.

In experiments on rats with liposomally entrapped ^{125}I-insulin or ^{14}C-inulin the proportion of the injected dose of tracer recoverable by excision of the injection site remained constant after about 1 h and 70% of the dose was still fixed in subcutaneous tissue for at least 5 h thereafter. When the plasma collected 3 h after subcutaneous injection of labeled liposomes containing ^{125}I-insulin was passed through a column of Sepharose 6B, 50–75% of the ^{125}I-activity was found in the fractions associated with intact liposomes. One possibility for the persistence of the hypoglycemic effect and of measurable IRI following injection of liposome insulin could be the presence of intact liposomes in the circulation for many hours after absorption had ceased. DIABETES *31*:506–511, June 1982.

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Liposomes (phospholipid vesicles consisting of concentric lipid bilayers enclosing aqueous compartments[1]) can be used as a carrier system to facilitate the parenteral administration of therapeutic agents such as hormones, anti-tumor drugs, antibiotics, and enzymes. By this means the entrapped material is not exposed to the circulation and can be "homed" specifically to the liver, this tissue being the natural target for liposomes.[2–4] In view of recent evidence that there are qualitative and quantitative differences in the metabolic responses to intravenous infusion of insulin by the portal and peripheral circulations in normal and diabetic dogs,[5–8] selective application of insulin to the liver may be necessary to restore all insulin-modulated responses to normal.[9] The present study investigated the potential of liposomes as a carrier for insulin by assessing the biologic responses produced by subcutaneous injection of insulin entrapped in liposomes to diabetic dogs.

In parallel studies using rats, the fate of injected liposomes and their contents was studied by the use of tracers. After subcutaneous injection of ^{125}I-insulin entrapped in liposomes, the disappearance of radioactivity from the site of injection and the appearance in the plasma of intact liposomes still containing radioactivity were estimated. Liposomes with ^{3}H-cholesterol in the lipid bilayer and the labeled polysaccharide ^{14}C-inulin in the aqueous compartment were also used to follow the disappearance of the liposomes and their contents from the subcutaneous injection site and their appearance in the plasma and liver. Inulin was used as a marker as it is a nonmetabolized polysaccharide of similar molecular weight to insulin and its properties make it an ideal aqueous marker to demonstrate the structural integrity of liposomes in the body.[10]

MATERIALS AND METHODS

Egg phosphatidyl-choline (lecithin) was obtained from Lipid Products (Epsom, United Kingdom); cholesterol from Sigma (St. Louis, Missouri); dicetylphosphate from K & K Laborato-

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ries; phosphatidyl [N-methyl-^{14}C]choline, [^{14}C-inulin]carboxylic acid, ^{125}I-insulin, and [1α, 2α(n)-^{3}H]cholesterol from The Radiochemical Centre (Amersham, United Kingdom). Novo monocomponent porcine insulin for incorporation into liposomes was a gift from Dr. W. R. Buckett (Novo Industri, Copenhagen, Denmark). Trypsin was obtained from Flow Laboratories Ltd. (United Kingdom). Sepharose 6B and DEAE Sephadex A-25 were purchased from Pharmacia Fine Chemicals AB (Sweden).

ANIMALS AND EXPERIMENTAL PROCEDURE

Dog. Male beagle dogs weighing 13–17 kg were prepared with indwelling venous cannulae as described previously[5] and diabetes was induced using alloxan and streptozotocin.[11] Blood glucose was controlled by daily injections of monocomponent porcine insulin (Actrapid and Ultralente, Novo Industri) since this is identical to canine insulin.[12] Ultralente and Actrapid were withheld 48 and 24 h, respectively, before the start of each experiment. Each dog was fasted overnight, and 1-ml heparinized venous blood samples were withdrawn for up to 9 h and again at 24 h after subcutaneous injection of 0.3–0.4 ml liposomes (≡75–100 mg lipid) containing insulin (0.75 U/kg) or the control solutions of either free insulin (Actrapid, 0.75 U/kg) or free insulin plus empty liposomes. The plasma was analyzed for glucose using the Beckman Analyzer (Beckman Instruments, Fullerton, California), which employs glucose oxidase. The remainder was frozen for subsequent double-antibody radioimmunoassay of insulin (kit from Radiochemical Centre, Amersham).

Rat. Male or female Wistar rats (200–225 g) were injected subcutaneously with 0.2 ml liposomes (≡25 mg lipid) containing ^{125}I-insulin or with liposomes (labeled with ^{3}H-cholesterol) containing ^{14}C-inulin. The control solution of ^{14}C-inulin with empty ^{3}H-cholesterol labeled liposomes was injected in separate groups of rats. Groups of six rats were killed at various times after injection and the tissue surrounding the injection site excised and homogenized in 4 ml 0.1 mM phosphate saline buffer, pH 7.4. After centrifugation the supernatant was counted for ^{125}I or ^{3}H and ^{14}C, respectively.

The ^{14}C- and ^{3}H-radioactivity in the blood, liver, and other tissues was measured following digestion of each tissue by 33% KOH (4 ml/g tissue) for 1 h at 70°C. The sample (100 μl) was neutralized with 1 ml of 1.5 mM HCl before addition of toluene scintillant containing Triton X-100.[13]

Plasma (0.5 ml, pooled from two rats) collected 3 h after subcutaneous injection of liposomes (labeled with ^{3}H-cholesterol) containing ^{125}I-insulin was passed through a column (27 × 1.5 cm) of Sepharose 6B to estimate the proportion of free to liposome-associated ^{125}I-radioactivity.

^{125}I was counted in a Packard Gamma Scintillation Spectrometer Model 49-26 and ^{3}H and ^{14}C in a Packard Liquid Scintillation Counter. An equal volume of each solution injected was counted for radioactivity after treatment as above and all results expressed as a percentage of the total injected dose.

Preparation of liposomes. Preparation of liposomally entrapped insulin has previously been described in detail.[14] Liposomes were composed of phosphatidylcholine (lecithin) and cholesterol with dicetylphosphate (for negative charge) or without it (for neutral charge) in a ratio of 10:2:1 by weight. The insulin liposomes were prepared by handshaking after adding 0.4% (w/v) insulin solution in 5 mM phosphate buffer, pH 7.4 (containing a trace of ^{125}I-insulin), to a dry film of lipid mixture. Liposomes used in these studies were not sonicated and are hence referred to as "handshaken" liposomes. They were large and multi-lamellar with an average diameter of 98 nm (range 30–270 nm).[15] The liposomally entrapped insulin was separated from free insulin by centrifugation at 120,000 *g* for 30 min. The resulting liposome pellet was washed, suspended in phosphate-buffered saline, pH 7.4, and recentrifuged. The final liposome pellet was washed and then resuspended in a minimum amount of phosphate-buffered saline, pH 7.4, the final concentration of lipid being 250–300 mg/ml. The amount of insulin entrapped and nonspecifically associated with the liposomes was determined by measuring ^{125}I-radioactivity. The amount of 0.3–0.4 ml of this preparation containing insulin (entrapped or associated with the surface of the liposomes) was injected subcutaneously to the diabetic dogs at a dose of 0.75 U/kg.

In some experiments to be described, the insulin liposome preparation was exposed to trypsin to remove insulin nonspecifically associated with the external surface of the liposomes. The liposome pellet was suspended in 3 ml phosphate saline buffer, pH 7.4, and 2 ml 0.5% trypsin was added. The mixture was incubated for 30 min at 37°C and the liposomes were recovered by centrifuging at 120,000 *g* for 30 min followed by washing and recentrifuging the pellet twice with phosphate-saline buffer, pH 7.4.

Iodinated insulin. As the experiments using rats required ^{125}I-insulin of higher specific activity than that available commercially, insulin was iodinated using the iodogen method of Salacinski et al.[16] Insulin (5 μg) and Na^{125}I (1 mCi) were added in 0.2 M phosphate buffer, pH 7.4, to a film of iodogen reagent (2.5 μg) deposited in a small glass tube, and incubated for 20 min. The final reaction mixture was applied to a 1 × 20-cm column of DEAE Sephadex A-25 equilibrated with buffer. Unreacted ^{125}Iodide was retained on the top of this column and the iodinated peptide emerged as a single broad peak.

RESULTS

Subcutaneous (s.c.) administration of liposomes containing insulin. Injection of the control solution of free insulin (0.75 U/kg) with empty liposomes produced a mean (± SEM) immunoreactive insulin (IRI) concentration of 510 ± 21 μU/ml in the plasma of five diabetic dogs 1 h after s.c. injection, levels that returned virtually to control values within 3 h (Figure 1). This insulin peak resulted in a sharp fall in plasma glucose from 16.4 ± 0.8 mmol/L to 2.9 ± 0.4 mmol/L 2 h after the injection and rose to 23.0 ± 0.6 mmol/L by 24 h. Subcutaneous injection of free insulin (Actrapid) alone produced an identical response (not shown).

In contrast, s.c. injection of insulin (0.75 U/kg) in negatively charged liposomes produced plasma IRI concentrations (measured in frozen and thawed plasma in an attempt to disrupt any intact liposomes) that peaked at 29.0 ± 4.5 μU/ml 1 h after injection. Thereafter they remained at a mean level of 15.5 ± 0.8 μU/ml from 2 to 9 h after injection and were still detectable in the diabetic dog after 24 h (Figure 2). A sharp fall was observed in plasma glucose concentration from 16.2 ± 0.4 mmol/L to 4.2 ± 0.4 mmol/L 2 h

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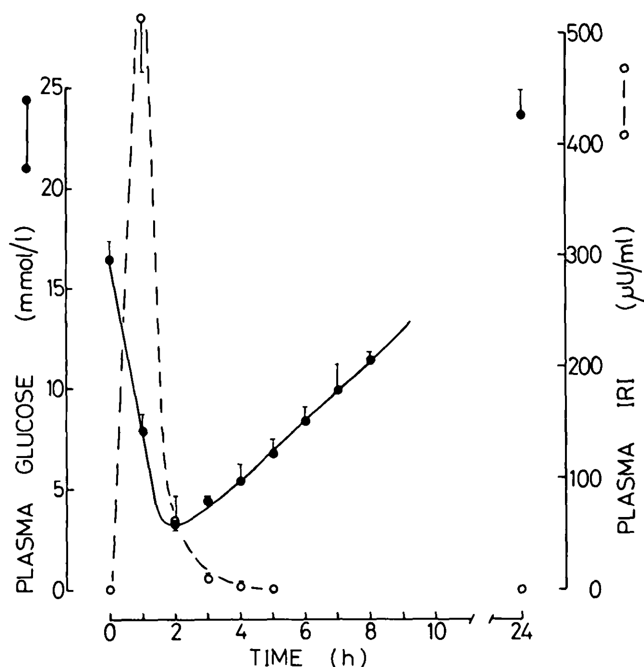

FIGURE 1. The effect of s.c. injection of free insulin (0.75 U/kg) with empty liposomes (≡75–100 mg lipid), at time zero, on mean (± SEM) plasma levels of immunoreactive insulin (IRI) (O--O) and glucose (●—●) in five diabetic dogs.

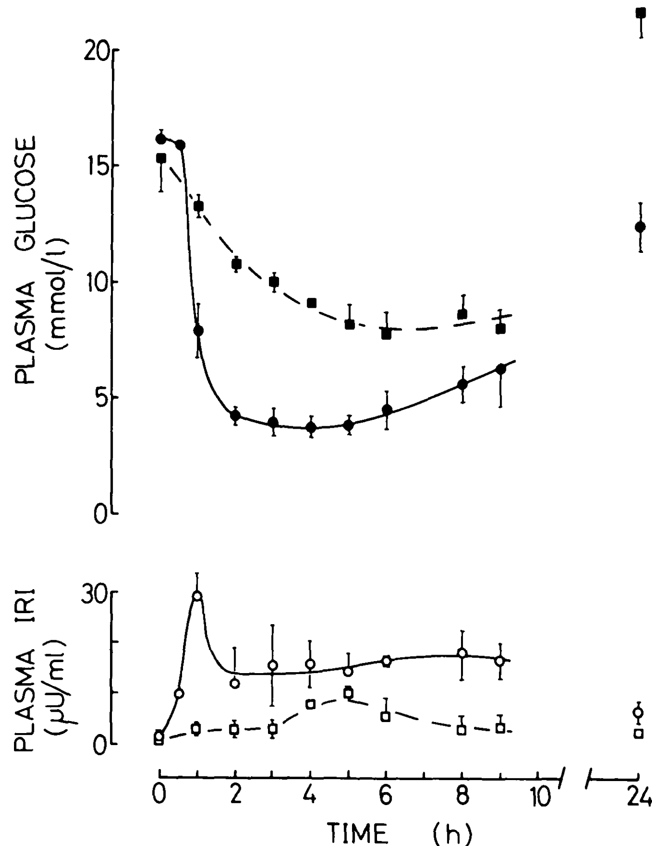

FIGURE 2. The effect of s.c. injection, at time zero, of insulin (0.75 U/kg) entrapped in and associated with negatively charged liposomes on mean (± SEM) plasma levels of immunoreactive insulin (IRI) (O—O) and glucose (●—●) in five diabetic dogs. Mean plasma levels of insulin (□--□) and glucose (■--■) in three diabetic dogs produced by s.c. injection of the same insulin liposomes pretreated with trypsin are also shown.

later and low levels were maintained for at least 9 h after injection. Plasma glucose had still not returned to initial levels even 24 h after the injection. When a similar experiment was performed using neutral liposomes (see METHODS), a much smaller biologic response was obtained. When the negatively charged insulin-liposomes were exposed to trypsin digestion, approximately 25% of the total insulin was removed. Plasma IRI levels resulting from s.c. injection of trypsin-treated insulin-liposomes remained relatively low throughout, and the glucose response, though less than with the untreated preparations, lasted at least 9 h (Figure 2).

Disappearance from the site of injection and appearance in blood and tissues. When the s.c. injection site was exposed at various times after injection of liposomes to rats, the injectate appeared diffuse for the first hour but thereafter the liposomes aggregated to form a distinct pellet. Therefore, total recovery of the injectate from the s.c. injection site at the earlier time points may not have been as efficient and may have produced slightly lower results than the true values.

When liposomes incorporating two separate radioactive tracers (one in the lipid bilayer and the other in the aqueous compartment) were injected s.c. in rats, disappearance of about 25% of the aqueous marker (either ^{125}I-insulin or the polysaccharide ^{14}C-inulin) from the injection site occurred over approximately the first hour. Thereafter it remained fairly constant till 5 h, though slow absorption of relatively small amounts of aqueous marker may still be occurring (Figure 3). About 40% and 70% of the injected dose of ^{125}I-insulin and ^{14}C-inulin, respectively, were still fixed in subcutaneous tissue at 24 h. In contrast, when free ^{125}I-insulin or the polysaccharide was injected with empty liposomes, 90% had disappeared by 2 h. Disappearance of the ^{3}H-labeled cholesterol was indistinguishable whether injected as empty liposomes or as inulin entrapped liposomes (Figure 3).

After s.c. injection of liposomes containing ^{14}C-inulin, low levels of the polysaccharide were seen in the blood for many hours, while a sharp peak was found within 1 h after injection of ^{14}C-inulin outside liposomes (Figure 4), resembling the patterns of plasma IRI observed following s.c. injection of free (Figure 1) and liposomally entrapped insulin (Figure 2). Empty and inulin-liposomes produced similar blood levels of ^{3}H-cholesterol.

Approximately 10% of the total injected dose of ^{14}C-inulin accumulated in the liver by 24 h after injection of either inulin-liposomes or empty liposomes plus free inulin (Figure 5). However, by 24 h only 30% of the entrapped inulin had disappeared from the injection site (Figure 3). Therefore, about 33% of the entrapped material absorbed into the circulation ended up in the liver. Accumulation of ^{14}C-inulin in the liver continued from 4 to 24 h after injection, suggesting that absorption of small amounts from the subcutaneous injection site may be occurring in this period.

When the plasma collected 3 h after s.c. injection of ^{3}H-labeled cholesterol liposomes containing ^{125}I-insulin was passed through a column of Sepharose 6B, 50.3% of the ^{125}I-activity and 65.5% ^{3}H-cholesterol were recovered in the void volume where intact liposomes are eluted[17] (Figure 6). The ^{125}I-activity eluted in the fractions corresponding to free insulin was 18.5%. About 25% of the ^{125}I-activity and ^{3}H-cholesterol was recovered from the top of the column where aggregates had formed. The results show that about 50–75%

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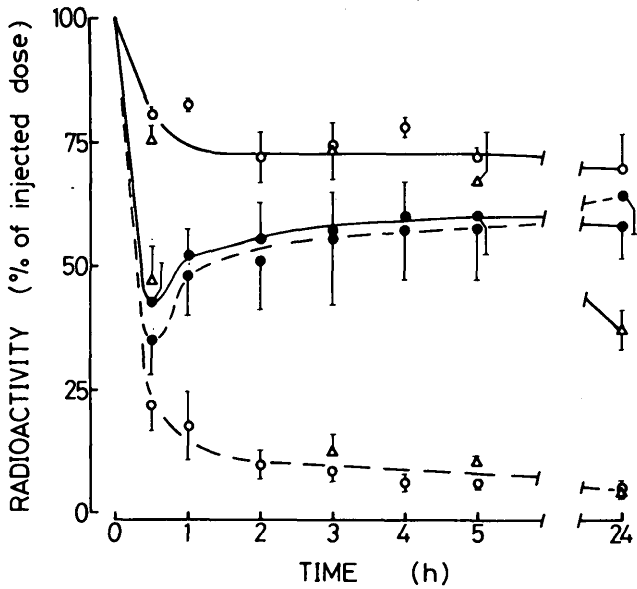


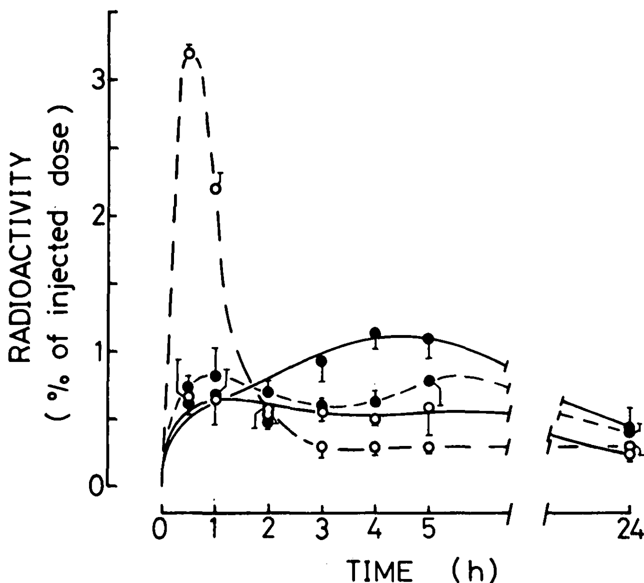
FIGURE 3. The radioactivity—^{14}C(○), ^{125}I (△), and ^{3}H(●)—measured at the site of injection (see METHODS) following s.c. injection into rats of: (1) ^{3}H-cholesterol labeled liposomes (≡25 mg lipid) (●—●) containing ^{14}C-inulin (○—○); (2) empty ^{3}H-cholesterol labeled liposomes (●-●) plus free ^{14}C-inulin (○--○); (3) liposomes containing ^{125}I-insulin (△—△); and (4) empty liposomes plus free ^{125}I-insulin (△--△). Results are expressed as the mean percentage (± SEM) of the total injected dose in groups of six animals.

of the ^{125}I-activity in plasma appeared to still be associated with intact liposomes.

DISCUSSION

These results show that subcutaneous injection of negatively charged liposomes containing insulin (0.75 U/kg) produced a much greater biologic response in diabetic dogs (measured as the area above the curve) than injection of the same amount of free insulin with or without empty liposomes. Indeed the glucose fall was extended by at least 7 h when the insulin was entrapped in liposomes, and a small response still remained 24 h after injection (Figure 2).

FIGURE 4. The radioactivity—^{14}C(○) and ^{3}H(●)—measured in the blood collected from rats injected subcutaneously with ^{3}H-cholesterol labeled liposomes containing ^{14}C-inulin (—) or empty ^{3}H-cholesterol labeled liposomes plus free ^{14}C-inulin (---) as described in Figure 3. Results are expressed as the mean percentage (± SEM) of the injected dose in groups of six animals. The blood volume of each rat was assumed to be 57.5 ml/kg body wt.[23]



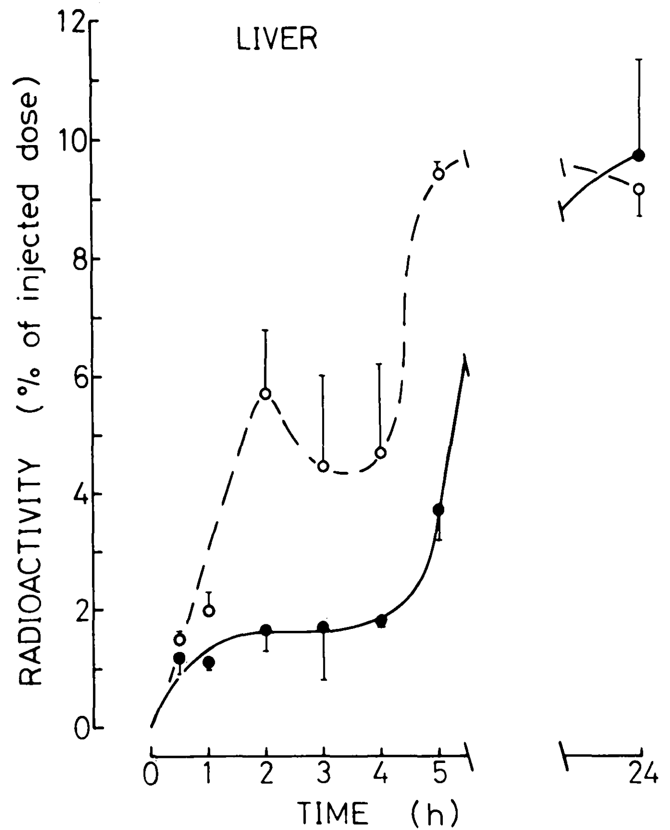
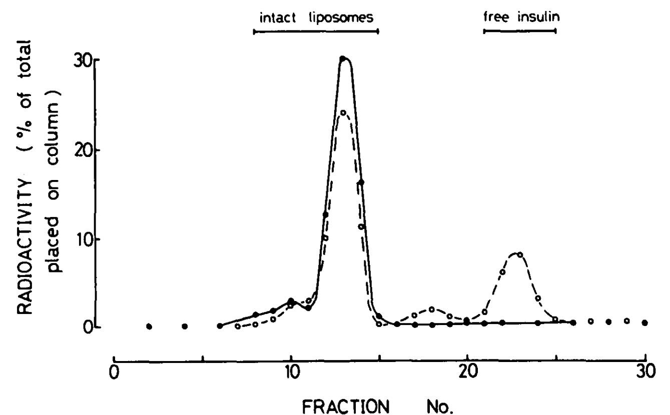


FIGURE 5. The radioactivity accumulating in the livers of rats injected subcutaneously with liposomes containing ^{14}C-inulin (●—●) or empty liposomes plus free ^{14}C-inulin (○—○). Results are expressed as the mean percentage (± SEM) of the injected dose in groups of six animals.

Addition of 100% excess lipid to the liposomes (≡150 mg lipid) before injection produced a response indistinguishable in duration and pattern from that following subcutaneous injection of the original liposome preparation (H. M. Patel and R. W. Stevenson, unpublished observations), confirming that the presence of lipid per se is not responsible

FIGURE 6. Elution profile of ^{125}I (○--○) and ^{3}H (●—●) from column of Sepharose 6B after addition of 0.5 ml plasma (pooled from two rats) collected 3 h after s.c. injection of ^{125}I-insulin entrapped in negatively charged liposomes (labeled with ^{3}H-cholesterol). The results are expressed as the percentage of the total radioactivity placed on the column.



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for slowing the rate of absorption of insulin into the circulation.[18] The early phases of the hypoglycemic response were probably due to the presence of insulin nonspecifically associated with the external surface of the liposomes and was reflected in a small peak of plasma IRI 1 h after injection (Figure 2). In addition, removal of the external insulin by trypsin digestion resulted in a slower onset of action and a reduced magnitude of the glucose fall, though this was still maintained for many hours (Figure 2). Although the liposomes were centrifuged and washed to remove trypsin, it is still uncertain whether trace amounts of trypsin remained associated with the surface of the liposomes. The later phases of the hypoglycemic response are probably the result of the presence in the circulation of immunoreactive insulin (measured in frozen and thawed plasma).

In another series of experiments using rats, liposomes incorporating separate tracers for the lipid (^{3}H-cholesterol) and contents (^{14}C-inulin) were injected subcutaneously. Low levels of ^{14}C were seen in the blood for many hours, resembling the appearance of immunoreactive insulin (IRI) described in dogs. The appropriateness of the tracer for following the fate of liposomally entrapped material such as insulin is also supported by comparing the sharp peak of IRI after injection of free insulin with the appearance in plasma of ^{14}C following injection of ^{14}C-inulin with empty liposomes (Figure 4). ^{14}C-inulin was chosen as a marker for these studies because (1) it is of similar molecular weight to insulin, (2) it is not metabolized, (3) free inulin is not taken up by the tissues in significant amounts, (4) it does not readily leak out of liposomes, and (5) its nonspecific association with the external surface of the liposomes is minimal,[10] making it easier than with insulin to dissociate the fate of the liposomal contents from any material associated with the external surface. This latter property explains why the initial IRI peak, seen 1 h after s.c. injection of insulin liposomes (Figure 2), was not observed when ^{14}C-inulin was employed (Figure 4).

While 90% of the free ^{14}C-inulin or ^{125}I-insulin injected with empty liposomes had disappeared from the injection site 2–3 h after s.c. injection in rats, only 25% of ^{14}C-inulin or ^{125}I-insulin entrapped within liposomes had disappeared by this time (Figure 3). Disappearance of ^{3}H-labeled cholesterol was indistinguishable whether injected as empty liposomes or as inulin-filled liposomes. However, the absolute amount of liposomes per se remaining at the injection site cannot be accurately determined since labeled cholesterol exchanges with unlabeled cholesterol from surrounding cells.[24]

The evidence indicates that s.c. injection of liposome insulin produces a prolonged hypoglycemic response even though a substantial portion of the total injected dose is still fixed in subcutaneous tissue 24 h after injection. Other reports confirm our observation that large proportions of intact handshaken liposomes remain at the injection site more than 24 h after injection,[15,18,19] and since a greater proportion of neutral than negatively charged liposomes remains at the injection site,[18] this probably explains why insulin entrapped within negatively charged liposomes produced the greater biologic response.

The prolonged hypoglycemic response in diabetic dogs may be partly due to the presence of intact liposomes in the circulation. This suggestion was supported by our experiments with entrapped ^{125}I-insulin indicating that about 50–75% of the absorbed insulin is associated with intact liposomes in the circulation 3 h after s.c. injection (Figure 6), i.e., 2 h after the relatively fast absorption from the injection site of about 25% of the total injected dose had slowed considerably (Figure 3). Although there is some disintegration of liposomes in the circulation,[20] it appears that the majority of insulin liposomes remain intact and have the ability to lower blood glucose. As the structural integrity of the liposomes was preserved, it appears that liposomes may protect insulin from degradation at the subcutaneous site[21,22] and in the circulation following absorption.

Since intact liposomes are preferentially taken up by the liver, the amount of entrapped material reaching the liver was measured. About 33% of the total amount of entrapped ^{14}C-inulin absorbed into the circulation accumulated in the liver by 24 h after s.c. injection. Since the amounts taken up by muscle and fat are very much less than that taken up by liver, even after intravenous injection,[10] the possibility exists that the significant hypoglycemia persisting for many hours after absorption of these small quantities of insulin reflects a direct hepatic action of insulin liposomes. To test this hypothesis a further study is in progress employing isotopically labeled glucose using the techniques described elsewhere.[8]

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