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TRANSPORT OF SULFONATED TETRAPHENYLPORPHINE BY LIPOPROTEINS IN THE HAMSTER

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Abstract—We have examined the transport and distribution properties of a bisulfonated tetraphenylporphine (TPPS-2A), an amphiphilic photosensitizer that spontaneously associates to lipoproteins. At different times after intravenous injection of TPPS-2A in hamsters, plasma was fractionated by density ultracentrifugation and porphyrin concentrations were measured in the different plasma (lipo)protein fractions. In order to mimic human lipoprotein composition hamsters were preinjected with 23 mg of apolipoprotein/kg human low density lipoprotein. In addition, the whole body distribution is described as detected by a novel method for the tissue quantification of TPPS-2A. At 5 min after injection into the penile vein, more than 50% of the injected dose appears to be associated with lung tissue, while only 30% is present in plasma and bound exclusively to plasma lipoproteins. After the initial phase, a more retarded decrease in plasma porphyrin concentration is observed. Between 5 min and 6 hr after administration, a redistribution of TPPS-2A from the lungs to the liver takes place. It is concluded that in the hamster, an animal model representing human lipoprotein composition, TPPS-2A is transported essentially exclusively by plasma lipoproteins. No depletion or accumulation of TPPS-2A in a particular plasma lipoprotein fraction could be observed, suggesting a continuous redistribution of the compound. The molecular skeleton of TPPS-2A may serve as a model for the development of new drugs that either have improved *in vivo* properties due to transport by lipoproteins or have a beneficial effect on the lipoprotein particle itself.

Albumin is well known as a blood constituent that can bind foreign compounds. Far less explored is the role of blood lipoproteins in the transport of exogenous molecules [1]. A few toxicological and pharmacological compounds have been reported to associate to lipoproteins: benzo(*a*)pyrene [2], testosterone undecanoate [3] and cyclosporin A [4] were reported to bind to serum high density lipoprotein (HDL†) and low density lipoprotein (LDL).

Recently a number of commercially available porphyrin preparations were found to associate with lipoproteins both *in vitro* and *in vivo* [5–8]. It has been suggested that transport of porphyrins, especially by LDL, may be related to the observed accumulation of these compounds in neoplastic tissues [7, 9]. Most preparations studied, however, are poorly resolved mixtures of mono-, di-, trimeric (and higher) aggregated species of porphyrins and, up till now, little is known about the structural requirements necessary for incorporation into lipoproteins. In the present investigation, we have examined a series of synthetic sulfonated tetraphenylporphines for their affinity for LDL. Their structures are presented in Fig. 1. The derivative with two adjacent sulfonate moieties (tetraphenylporphine sulfonate, TPPS-2A), showed the best incorporation characteristics. We have subsequently focused our attention on the fate of TPPS-2A *in vivo*, especially with regard to its association with the different lipoproteins. The current data may be useful in the development of new classes of pharmacological compounds that aim to associate to lipoproteins.

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† Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein; LPDS, lipoprotein deficient serum; TPPS, tetraphenylporphine sulfonate (the number following denotes the number of the sulfonate group; in the case of TPPS-2A, two adjacent); PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide.

MATERIALS AND METHODS

Materials. Tetraphenylporphine (TPP) and its sulfonated analogues (TPPS-1, TPPS-2A and TPPS-4) were obtained from Porphyrin Products Inc. (Logan, UT, U.S.A.). Dimethylsulfoxide (DMSO) and potassium bromide were purchased from J. T. Baker (Deventer, The Netherlands). Agarose was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Precilip, cholesterol oxidase, cholesterol esterase and the cholesterol Boehringer kit 310328 was from Boehringer Mannheim (Mannheim, Germany). All fluorescence measurements were performed on a Perkin–Elmer LS-5B luminescence spectrometer.

Lipoproteins. LDL was isolated from human plasma at density $1.024 < d < 1.055$ g/mL by two repetitive centrifugations according to Ref. 10 as previously described [11]. The LDL preparation dissolved in phosphate-buffered saline (PBS) (pH 7.4) contained solely apoprotein B (99.97%) and no degradation products were noticeable when checked by electrophoresis in sodium dodecyl sulfate (SDS) gels.

Determination of log P of the porphyrins. Log *P* was determined according to a modification of the procedure described by Yeh and Higuchi [12] and

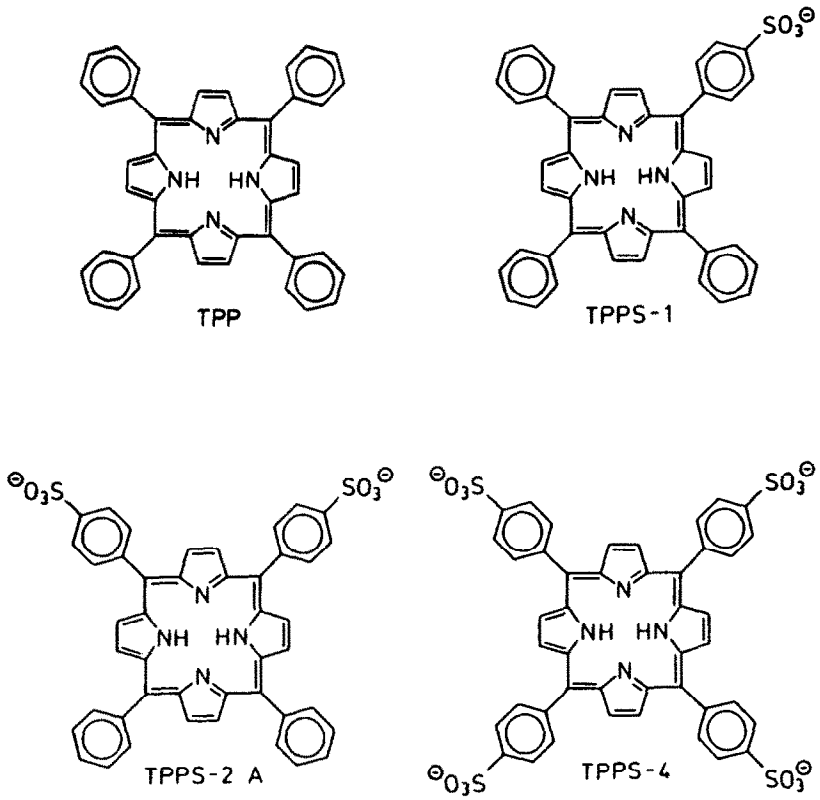

Fig. 1. Chemical structures of TPP, TPPS-1, TPPS-2A and TPPS-4.

Hobbelen *et al.* [3]. Twenty-five nanomoles of the porphyrins were coated on 4 mL stoppered glass vials. Octanol (1.0 mL) and 1.0 mL of PBS–1 mM EDTA, pH 7.4 were added and the samples were incubated on a Luckham Multimix MM1 rollerbank for 24 hr at room temperature. Samples of octanol and the water phase were then assayed for fluorescence at 415 and 630 nm (excitation and emission wavelength, respectively).

Determination of incorporation in LDL. The assay is based upon the "dry film" incorporation procedure [13]. Twenty-five nanomoles of TPP, TPPS-2A or TPPS-4 were coated on a glass tube by evaporation under N_2. Subsequently, 520 μg (protein, 1 nmol) of LDL was added and all samples were incubated for 3 hr at 37° under N_2 in the dark. To separate LDL-bound from unbound porphyrins, samples of the preparations were subjected to agarose gel electrophoresis. After the electrophoresis was complete, the gel was cut in slices and subsequently extracted quantitatively with PBS–EDTA. All extracts were then assayed for porphyrin by fluorescence measurement at 415 nm (excitation) and 650 nm (emission). Values are expressed as the fraction of the total available amount (25 nmol) that was incorporated in LDL.

Plasma decay and plasma distribution. Male Syrian hamsters (85–105 g) were anesthetized with Nembutal. Ten minutes before the administration of 5 mg/kg TPPS-2A (in PBS pH 7.4), the animals were preinjected with 23 mg of apolipoprotein/kg LDL in order to obtain an animal model with plasma LDL/HDL ratios more related to the human situation. Both injections were administered in the penile vein.

Of each hamster two or three samples of 0.5 mL blood were taken by orbital puncture at indicated time points after injection of TPPS-2A. The blood samples were centrifuged (10 min at 10,000 *g*) and 200 μL plasma was isolated and transferred to a polyallomer centrifuge tube. Thereafter, 1106 mg of solid KBr and PBS–1 mM EDTA were used for the complete dissolution of KBr to a final volume of 4.0 mL. Consecutive layers of 3.0 mL of KBr (1.063, 1.019 and 1.0063 g/mL, respectively) were then added. After centrifugation, 500 μL samples, starting from the bottom of the tube, were taken and the gradient was subdivided according to density. Samples (200 μL) were then mixed with 1.8 mL DMSO, centrifuged and the supernatant was then measured for fluorescence at 415 and 650 nm (excitation and emission wavelength, respectively). In this experimental set-up, control experiments showed that KBr and proteins did not influence porphyrin fluorescence.

For the cholesterol assay, 100 μL of LDL- and HDL-containing fractions were assayed for free cholesterol using Boehringer kit 310328. Esterified cholesterol was determined by adding 20 μg cholesterol esterase after determination of free cholesterol. Precilip El.^{R} (4.04 μg cholesterol/μL) was used as an internal standard.

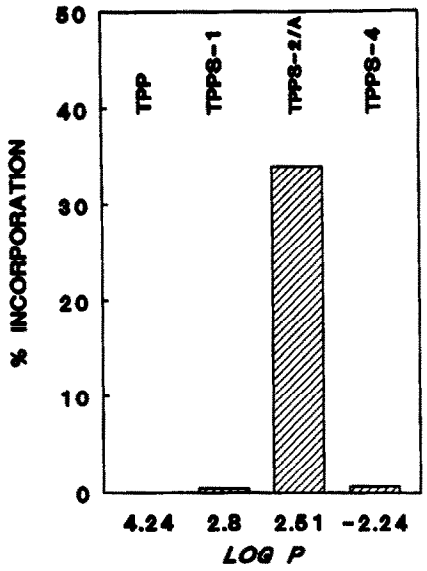

Fig. 2. Efficiency of incorporation of sulfonated tetraphenylporphines into LDL in relation to the octanol/H_2O distribution. Compounds (25 nmol) were incubated with a solution of 1 nmol of LDL according to the dry film procedure [13]. After incubation for 3 hr at 37°, samples were subjected to agarose gel electrophoresis. The LDL-containing segments in the gels were excised and measured for fluorescence. In addition, the octanol/water distribution of the compounds was determined (expressed as log *P*).

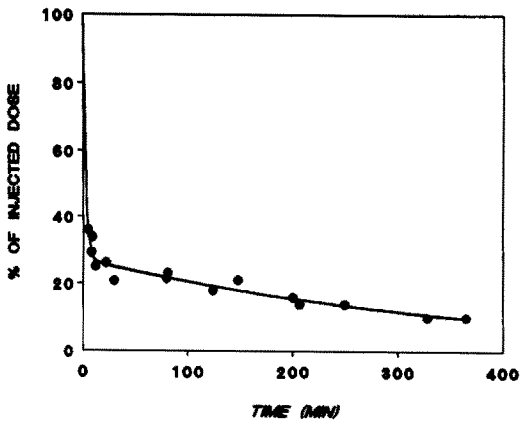

Fig. 3. Plasma decay of TPPS-2A in the hamster. Male Syrian hamsters were given a bolus injection of 5 mg/kg TPPS-2A in the penile vein. At the indicated times after injection, blood samples were collected, fractionated according to density and analysed for TPPS-2A fluorescence. Values represent the sum of TPPS-2A recovered in the different plasma fractions from a total of six hamsters.

Tissue distribution of TPPS-2A. Male Syrian hamsters (85–105 g) were anesthetized with Nembutal and preinjected in the penile vein with 23 mg of apolipoprotein/kg LDL. After 10 min, 5 mg TPPS-2A/kg (in PBS pH 7.4) was administered in the same injection site. Five minutes or 6 hr after the injection of TPPS-2A, the hamsters were killed and blood, urine and tissue samples were taken and weighed. After centrifugation of the blood sample, 50 μL of plasma was pipetted to 1.95 mL DMSO–10% PBS, 50 μL of the blood cell fraction was mixed with 50 μL 5% Triton X-100 and added to 1.9 mL DMSO–10% PBS. After centrifugation an aliquot of supernatant and diluted with DMSO–10% PBS and measured for fluorescence on a Perkin–Elmer LS-5B luminescence spectrometer at 415 nm (excitation) and 650 nm (emission) as described. For quantification of the amount of TPPS-2A in all tissue and blood samples, three consecutive 10 μL aliquots of 0.01 mg/mL TPPS-2A standard were added in the sample (standard addition procedure).

Urine: 100 μL urine was treated with 100 μL 5% Triton and 1.8 mL DMSO–10% PBS. After centrifugation, samples of the supernatant were diluted with DMSO–10% PBS and measured as described above.

Tissue samples: samples of liver, lung, heart, spleen and kidney (0.1–0.4 g) were homogenized in a total of 5.0 mL PBS. Homogenate (0.2 mL) was then mixed with 100 μL 5% Triton and 1.7 mL DMSO–10% PBS. After centrifugation, samples of the supernatant were diluted with DMSO–10% PBS and measured as described above. For the experiment shown in Fig. 8, the same procedure was followed except that the injection site was the portal vein instead of the penile vein.

All manipulations were carried out under reduced light exposure. All measurements showed excellent linearity at TPPS-2A concentrations in a range up to 0.3 μg/mL in the described solvent. Following this procedure, control experiments showed that 103% of administered porphyrins were recovered.

RESULTS

Incorporation efficiency of TPP, TPPS-1, TPPS-2A and TPPS-4 for LDL

In order to quantify the incorporation efficiency of the investigated series of tetraphenylporphine for LDL, porphyrins were incubated with LDL according to the dry film procedure [13]. In order to separate LDL-bound porphyrin from possible unbound porphyrins, the samples were subjected to agarose gel electrophoresis. Of the four tested porphyrins, TPPS-2A was found to possess the most favorable incorporation properties when compared with the other three compounds. Utilizing the dry film procedure, approx. 35% of the total amount coated on the glass surface incorporated into LDL (Fig. 2). Considering the data presented in Fig. 2, further experiments focused on TPPS-2A.

Fate of TPPS-2A in hamster plasma

Syrian hamsters were injected intravenously with TPPS-2A after pre-injection of 23 mg of apolipoprotein/kg human LDL. This amount of LDL was administered in order to obtain HDL/LDL ratios more comparable to the human situation [14] so that a better evaluation of the relative role of the various lipoprotein fractions in the transport of porphyrins could be obtained.

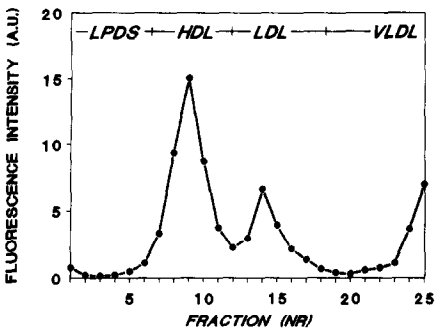

Fig. 4. Distribution of TPPS-2A between (lipo)protein fractions *in vivo*. At 80 min after injection, a blood sample was collected and plasma was fractionated by density ultracentrifugation. The different plasma density fractions were then analysed for TPPS-2A fluorescence. The presented pattern is also representative for analysed blood samples at other times after injection.

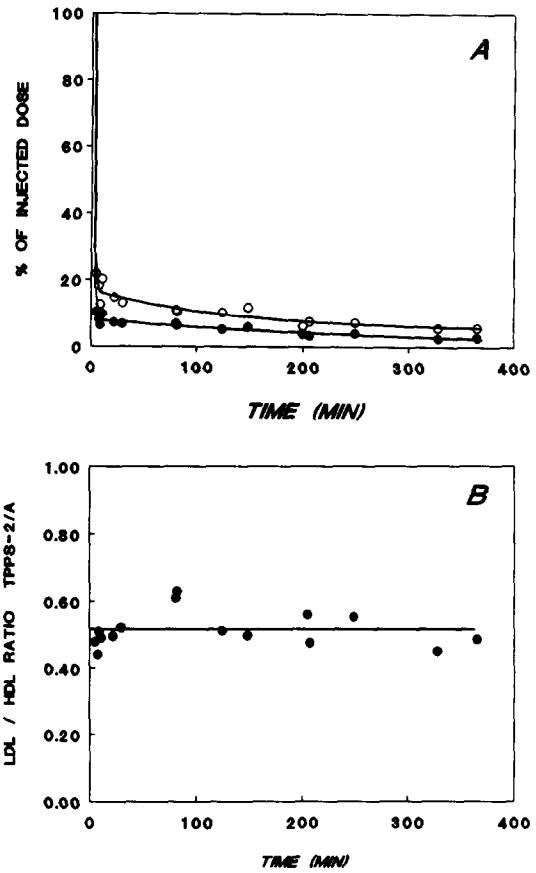

Fig. 5. Decay of TPPS-2A from the LDL and HDL fraction in hamster plasma *in vivo* (A) and relative distribution of TPPS-2A over LDL and HDL (B). After the injection of 5 mg/kg TPPS-2A, blood was collected and fractionated by density ultracentrifugation at various times after injection. The amounts of TPPS-2A in the HDL (○) and LDL (●) fractions of blood were then measured (A) and the recovery of TPPS-2A in LDL relative to HDL is presented in (B).

It was noted that within 5 min, more than 50–60% of the injected dose disappeared from the plasma compartment. After this apparent distribution phase, a more gradual decay of TPPS-2A fluorescence in the plasma is observed (Fig. 3), decreasing from 30%, 5 min after injection to 10–15% after 6 hr.

Plasma values presented in Fig. 3 are the total amounts of TPPS-2A recovered in plasma. After fractionation of the plasma and subsequent quantification of porphyrin in the different density fractions, most of the TPPS-2A fluorescence was recovered in the fractions with densities < 1.2 g/mL. Essentially no TPPS-2A fluorescence was observed in the lipoprotein deficient serum fraction ($d > 1.21$ g/mL) (Fig. 4). The distribution of fluorescence in plasma presented in Fig. 4 was from a single blood sample taken 80 min after injection. The pattern, however, with 3, 55, 27 and 15% of the fluorescence associated with respectively the LPDS, HDL, LDL and VLDL fractions was representative for all blood samples taken in this study.

The decay of TPPS-2A recovered in the HDL and LDL fractions of plasma during the investigated period (Fig. 5A) follows a similar pattern. Both for HDL and LDL, a gradual decrease in TPPS-2A fluorescence is observed, ranging from 20 to 7% for HDL and 10 to 4% for LDL. The distribution of TPPS-2A between HDL and LDL remains essentially constant during this period at a value of 0.52 (LDL/HDL) (Fig. 5B). The amounts of LDL relative to HDL in the blood compartment, as assessed by their cholesterol content, was 1.4 and was also relatively stable during the course of the experiment. It can be calculated that the observed relative distribution of TPPS-2A between LDL and HDL (34% in LDL, 66% in HDL) correlates better with the available lipoprotein surface areas (LDL: 26%, HDL: 74%) than with the core cholesterol content (LDL: 56%, HDL: 44%).

Tissue distribution of TPPS-2A

In order to evaluate the distribution of TPPS-2A in different body tissues, a procedure was developed based upon the excellent solubility of TPPS-2A in DMSO and the simultaneous precipitation of many tissue components as proteins and nucleic acids in this solvent.

Five minutes after injection of TPPS-2A, 53% of the dose is recovered in the lungs. Considerably less porphyrin is associated to plasma (25%), blood cells (4%) and liver (9%) (Fig. 6A). 6 hr after the injection, however, a significant reduction in the lung association to about 8% of the injected dose is measured (Fig. 6B). The amount associated with the liver has increased to 52%. The fraction of injected dose of TPPS-2A in heart, spleen and kidney is below 5%, while no significant TPPS-2A fluorescence in the urine is observed.

When expressed as micrograms of porphyrin per gram wet weight, it can be calculated that lungs rapidly bind TPPS-2A to reach values of 450 μg/g wet weight after 5 min (Fig. 7A). After 6 hr,

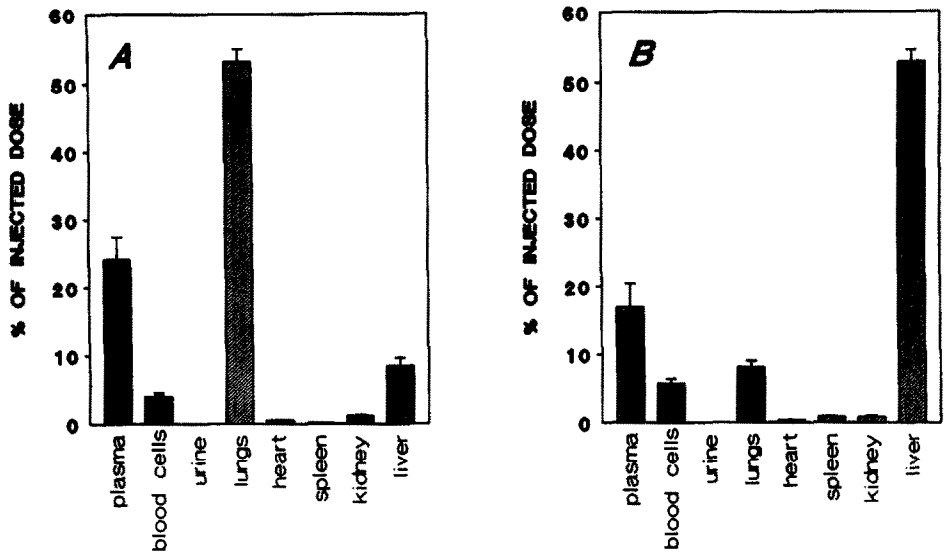

Fig. 6. Recovery sites of TPPS-2A 5 min (A) or 6 hr (B) after injection. At the indicated time after injection in the penile vein, the animals were killed and the various tissues were recovered, weighed and analysed for TPPS-2A fluorescence by extraction of tissue homogenates with DMSO as described in Materials and Methods. Values are expressed as per cent of the injected dose and are the average of three hamsters ± SEM.

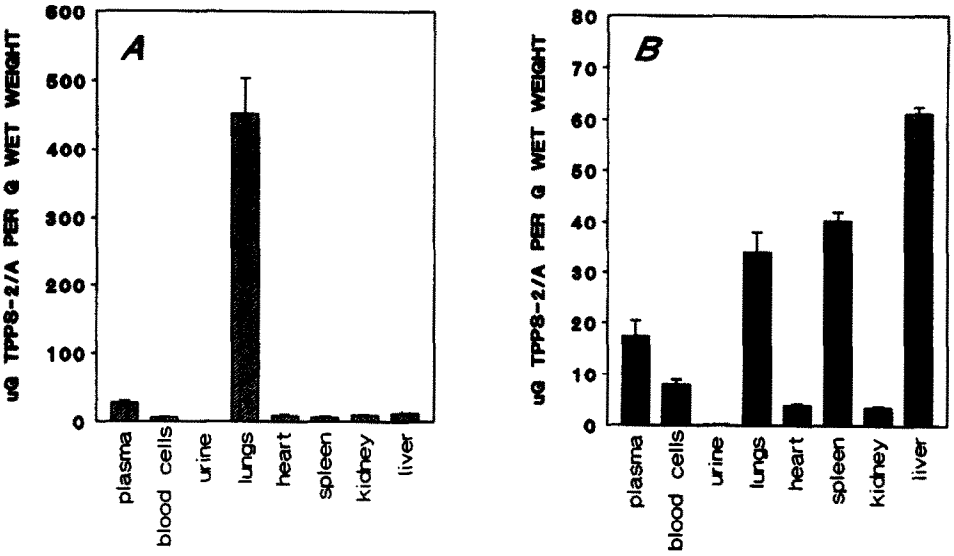

Fig. 7. Recovery sites of TPPS-2A 5 min (A) or 6 hr (B) after injection. At the indicated times after injection in the penile vein, the animals were killed and the various tissues were recovered, weighed and analysed for TPPS-2A fluorescence by extraction of tissue homogenates with DMSO as described in Materials and Methods. Values are expressed as μg TPPS-2A per g wet weight and are the average of three hamsters ± SEM.

this value was decreased to 33 μg/g. Spleen and liver show comparable associations of TPPS-2A (40 and 60 μg/g, respectively) while association to heart, kidney and urine does not exceed 5 μg/g (Fig. 7B).

It might be questioned to what extent the first pass effect may influence the observed prevalence for lung uptake of TPPS-2A. Injection of TPPS-2A in the portal vein led to a marked difference in tissue distribution (Fig. 8A and B). After 5 min, 78% of

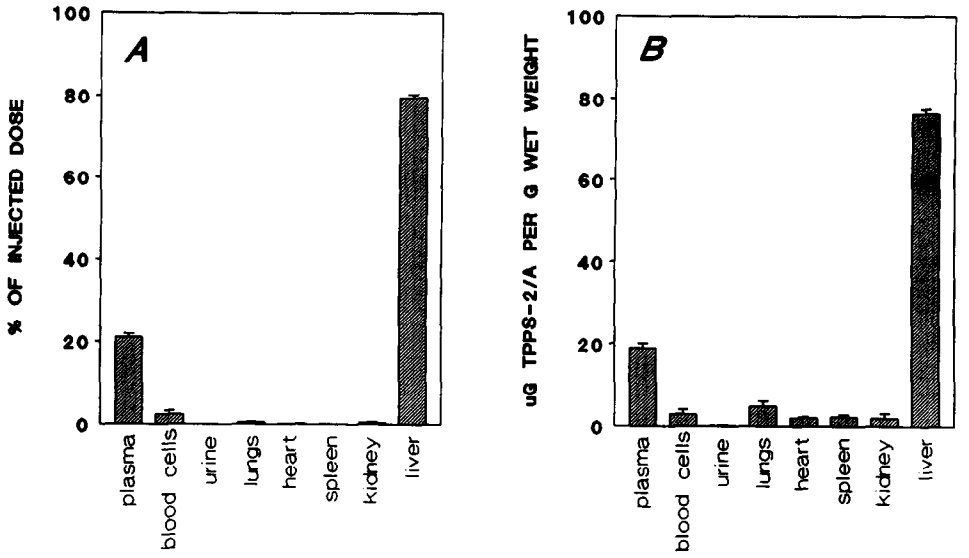

Fig. 8. Recovery sites of TPPS-2A at 5 min after injection in the portal vein. Five minutes after the injection of TPPS-2A in the portal vein, the animals were killed and the various tissues were recovered, weighed and analysed for TPPS-2A fluorescence by extraction of tissue homogenates with DMSO as described in Materials and Methods. Values are expressed as per cent of the injected dose (A) or as μg TPPS-2A per g wet weight (B) and are the average of three hamsters ± SEM.

the injected dose was recovered in the liver and only 0.5% was associated with lung tissue.

DISCUSSION

Previously, efforts have been made to incorporate drugs and other foreign compounds into lipoproteins, often by elaborate non-physiological procedures [15, 16]. In the present investigation, we have focused on a compound that spontaneously associates with lipoproteins, and in addition we have studied its behavior *in vivo*.

In a series of four tetraphenylporphines, TPPS-2A showed the highest incorporation efficiency in LDL. Up till now, the role of lipoproteins in the transport of TPPS-2A *in vivo* is largely unknown and we have therefore tried to follow the compound in the different plasma density fractions at various times after intravenous injection. To achieve an amount of LDL and HDL relevant to the human situation, a bolus (23 mg of apolipoprotein/kg) injection of LDL was administered in all animals prior to the porphyrin injection.

Within 5 min after injection of 5 mg/kg of TPPS-2A, a rapid decrease in the total amount of the porphyrin in plasma is observed. After this initial decrease, a much more retarded decay in plasma porphyrin concentration is measured. For all blood samples taken, TPPS-2A was associated almost exclusively with plasma lipoproteins, thus suggesting a major role for lipoproteins in the transport and (re)distribution of the compound. When the decay rate of TPPS-2A from HDL and LDL fractions is followed, a similar profile is observed, as the rapid decrease is followed by a much more retarded second phase in porphyrin decay. During the course of the experiment, HDL as compared to LDL transported approximately twice the amount of TPPS-2A. It can be calculated from the relative amounts of HDL and LDL in the hamsters and from the sizes of the two lipoproteins, that TPPS-2A does not preferentially bind to a specific lipoprotein but rather distributes over the available surface of lipoproteins. The relative distribution of TPPS-2A over LDL and HDL appeared thereby constant with a relative ratio of 0.52 (LDL–TPPS-2A : HDL–TPPS-2A).

A novel method was developed in order to measure the tissue distribution of TPPS-2A. The excellent solubility of TPPS-2A in DMSO allowed avoidance of the elaborate extraction procedures described for the tissue distribution studies of TPPS-4 [17]. Instead, DMSO extraction of diluted tissue homogenates followed by a standard addition procedure, showed excellent recovery, linearity and reproducibility. The first question we wanted to address was the decrease of plasma levels of TPPS-2A immediately after injection. The tissue distribution study indicated that 5 min after injection, more than 50% of the injected dose was associated with the lungs, resulting in a TPPS-2A uptake of almost 0.5 mg/g tissue. At 6 hr after injection, this value had decreased to 34 μg/g lung tissue and at this time point the lungs accounted for less than 10% of the injected dose. It appeared that TPPS-2A had redistributed and, at 6 hr after injection, the liver was responsible for the association of 53% of the amount of injected porphyrin. Regarding the complete absence of TPPS-2A in the albumin fraction of plasma *in vivo*, it might be suggested that lipoproteins play a major, if not the only role in the redistribution of TPPS-2A from lungs to liver. From the present investigation, it can be concluded that

TPPS-2A is transported *in vivo* essentially exclusively by lipoproteins.

The equal halflives of the compound in the LDL and HDL fractions of plasma suggest a continuous redistribution over plasma lipoproteins. It is not likely that the rapid association to lung tissue of over 50% of the injected dose can be explained by a specific process since injection in the portal vein resulted in a similar massive association with the liver. It may therefore be more reasonable to assume that TPPS-2A associates with the first major capillary network it encounters after intravenous injection.

It might then be anticipated that the intended tumor localization of porphyrins in the photodynamic therapy of cancer might be helped by the localized injection of this class of antineoplastic compounds. The excellent incorporation properties of TPPS-2A might also be utilized as a lipoprotein "anchor" for future drugs, for instance for antisense oligonucleotides [18] in the treatment of various genetic disorders. Alternatively, it can be regarded as a model compound for the development of new drugs that can bind to LDL and subsequently protect the lipoprotein from oxidative modification in order to avoid its accumulation in atherosclerotic plaques [19].

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