***O*-(4-Diazo-3,5-di[¹²⁵I]iodobenzoyl)sucrose, a novel radioactive label for determining organ sites of catabolism of plasma proteins**

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A method is described for radiolabelling proteins with *O*-(4-diazo-3,5-di[¹²⁵I]iodobenzoyl)sucrose (DD¹²⁵IBS). When proteins so labelled were degraded within lysosomes, the radioactive fragments were largely retained within the organelle. High specific radioactivities were obtained without changing the properties of the protein. The validity of the method was demonstrated *in vivo* in rats using the short-lived protein lactate dehydrogenase, isoenzyme M₄, and the long-lived protein bovine serum albumin. Derivatization with DD¹²⁵IBS did not alter the clearance of either protein. Uptake of DD¹²⁵IBS-labelled lactate dehydrogenase, isoenzyme M₄, by liver and spleen of rats was determined. Radioactivity in these tissues increased up to about 2 h after injection (at this time the protein has been almost completely cleared from the blood) and subsequently declined with a half-life of approx. 20 h. After differential fractionation of liver, radioactivity was largely found in the mitochondrial and lysosomal fraction. The results of these studies establish that DD¹²⁵IBS covalently coupled to plasma proteins should be a useful radioactive tracer for identifying the tissue and cellular sites of catabolism of relatively long-lived circulating proteins.

For the reliable assessment of the contributions of various tissues and cell types *in vivo* to the degradation of plasma proteins, a radioactive tracer is needed which will (1) not affect protein clearance and catabolism, (2) be resistant to degradation by lysosomal hydrolases, and (3) accumulate at the site of the labelled protein's degradation. The recently developed [³H]- or [¹⁴C]sucrose-containing labels (Pittman & Steinberg, 1978; Pittman *et al.*, 1979*a,b*; Van Zile *et al.*, 1979) meet these requirements. Sucrose is a carbohydrate which does not readily diffuse out of lysosomes (Horvat, 1973; Silverstein *et al.*, 1977; Wattiaux, 1977). Moreover, mammalian lysosomes do not contain significant levels of sucrase activity (Cohn & Ehrenreich, 1969; Horvat, 1973; Wattiaux, 1977) and consequently it is very unlikely that hydrolysis of the sucrose moiety in the label will occur before or after degradation of the protein. With ³H- and ¹⁴C-containing sucrose, however, the specific radioactivity obtainable is limited. Although high specific radioactivities can be obtained by conventional methods of iodination with ¹²⁵I, the tracer does not accumulate at the site of catabolism. Degradation of ¹²⁵I-labelled proteins leads to the formation of monoiodotyrosine, which diffuses very rapidly out of the lysosomes and the

cell. Since the rate of intracellular degradation within lysosomes far exceeds the rate of uptake of long-lived plasma proteins, ¹²⁵I is not a suitable label with which to monitor the sites of catabolism of these long-lived proteins. Using ¹²⁵I as radioactive tracer, the sites of catabolism of proteins with short half-lives (<30 min) can be readily identified since their rates of clearance are relatively rapid compared to the rate of lysosomal digestion. This has been shown for, e.g., ¹²⁵I-labelled ribonuclease B (Brown *et al.*, 1978; Schlesinger *et al.*, 1978), β -glucuronidase (Schlesinger *et al.*, 1978), asialo-orosomucoid (Schlesinger *et al.*, 1978), agalacto-orosomucoid (Schlesinger *et al.*, 1978) and lactate dehydrogenase, isoenzyme M₄ (Sinke *et al.*, 1979).

In this paper we describe the synthesis and use of a label (DD¹²⁵IBS) that contains both sucrose to prevent rapid diffusion of radiolabelled degradation products out of the lysosomes and ¹²⁵I to obtain a high specific radioactivity without changing the properties of the protein.

Experimental

Materials

Suspensions of pig lactate dehydrogenase, isoenzyme M₄, (EC 1.1.1.27) were obtained from Boehringer; Na¹²⁵I (carrier free) was from The

Abbreviation used: DD¹²⁵IBS, *O*-(4-diazo-3,5-di[¹²⁵I]-iodobenzoyl)sucrose.

Radiochemical Centre, 4-nitrobenzoyl chloride was from Aldrich, Sephadex G-10, DEAE-Sephadex A-25, DEAE-Sephacrose 6B-CL, and 5'-AMP-Sephacrose 4B were from Pharmacia, NADH and bovine serum albumin (fraction V) were from Sigma, sucrose was from BDH and Fluothane was from I.C.I. All other reagents were of analytical grade.

Preparation of $DD^{125}IBS$

4-Nitrobenzoyl chloride (1 mmol) was added in small portions to a stirred solution of 1 mmol of sucrose in 10 ml of pyridine (sucrose was dissolved by heating and the solution was dried over barium oxide before use) at 0°C. The reaction mixture was stirred for 3 h at 0°C and then allowed to stand at room temperature for 24 h. The solution was then cooled to 0°C and the reaction was stopped by the addition of 0.1 ml of water by stirring. After 30 min at 0°C, the pyridine and water were removed by rotary evaporation at 70°C. The residue was washed with three batches of 10 ml of water by suspending and rotary evaporation. The final residue was solubilized as far as possible in 10 ml of water and filtered. The filtrate was loaded onto a Sephadex G-10 column (2 cm × 45 cm) to isolate the monoester of 4-nitrobenzoylsucrose. The isolation is based on the fact that aromatic compounds are retarded on Sephadex compared with nonaromatic substances of similar size. The column was eluted with 0.1 M-ammonium acetate in water at a flow rate of 25 ml/h. Fractions (4.5 ml) were collected and aliquots were

analysed for A_{260} [the molar absorption coefficient of (4-nitrobenzoyl) $_n$ -sucrose at 260 nm is $14\,800 \times n$, in which n is the number of hydroxyl groups substituted in sucrose (Nachtmann, 1976)] and carbohydrate content (Fischer, 1974) with sucrose as standard. A qualitative ester test (hydroxamic acid test) on the fractions was carried out by the method of Vogel (1972). A typical chromatographic pattern is shown in Fig. 1. Fractions containing the monoester were pooled and lyophilized. The lyophilized material was dissolved in water and stored at -18°C. The molar ratio of the 4-nitrobenzoyl moiety to the sucrose moiety in the preparation was 1.

4-Nitrobenzoylsucrose was converted into 4-aminobenzoylsucrose by reduction with a 5-fold molar excess of sodium dithionite. A solution (0.5 ml) of 10 μ mol of 4-nitrobenzoylsucrose in 0.5 M-NaHCO₃ and 0.1 M-sodium dithionite (pH 8.5) was incubated at 37°C for 1 h and then loaded onto a DEAE-Sephadex A-25 column (counter ion, Cl⁻) (2 cm × 25 cm) to remove (bi)carbonate ions, dithionite ions and byproducts of the reaction (sulphite ions and 4-sulphoaminobenzoyl sucrose). The column was eluted with 150 ml of water at a flow rate of 25 ml/h. Fractions (4.5 ml) were collected and analysed for A_{290} , carbohydrate content (Fischer, 1974, with sucrose as standard) and aromatic amino content as described below. Fractions containing 4-aminobenzoylsucrose were pooled and lyophilized. The lyophilized material was dis-

Fig. 1. Chromatography of 4-nitrobenzoylsucrose on a column (2 cm × 45 cm) of Sephadex G-10 with 0.1 M-ammonium acetate as eluent

Flow rate was 25 ml/h. Fractions (4.5 ml) were collected and aliquots were analysed for A_{260} (O) and carbohydrate content (A_{625}) (●).

solved in water and stored at -18°C . A molar absorption coefficient of approx. 18000 at 290 nm was found for the monoester of 4-aminobenzoyl-sucrose. The molar ratio of the aromatic amino group to the sucrose moiety in the preparation was 1.

4-Aminobenzoylsucrose was radioiodinated with ^{125}I at a final pH of 4.7. To 50 μl of a solution of 20 nmol of 4-aminobenzoylsucrose in 1 M-NaCl and 0.3 M-sodium acetate buffer (pH 5.0) was added 25 μl of a ^{125}I solution (prepared by diluting 26.66 nmol of $\text{NaI} + \text{Na}^{125}\text{I}$, 13.34 nmol of KIO_3 , 25 μmol of NaCl and 2 μl of conc. HCl acid to 25 μl with water). After 24 h of reaction at room temperature, the reaction was stopped by the addition of 5 μl of 1 M-sodium bisulphite in water. The reaction mixture was loaded onto a DEAE-Sephacrose 6B-CL column (1 cm $\times$ 6 cm; counter ion, Cl^-) to remove unreacted iodide ions and (bi)sulphite ions. The column was eluted with water at a flow rate of 25 ml/h. Fractions (1 ml) were collected and analysed for radioactivity. Fractions containing *O*-(4-amino-3,5-di[^{125}I]iodobenzoyl)sucrose were pooled, lyophilized and stored at -18°C . Approx. 85–90% of the added iodide ions were incorporated, which is equivalent to an average incorporation of 1.7–1.8 atoms of iodine per molecule. A specific activity of at least 2000 Ci/mmol could be obtained. This value is much higher than the specific activities of commercially available [^3H]- and [^{14}C]sucrose (<5 Ci/mmol).

DD ^{125}IBS was prepared in the following way: to 0.1 ml of a solution of *O*-(4-amino-3,5-di[^{125}I]iodobenzoyl)sucrose in water were subsequently added 0.01 ml of 1 M- NaNO_2 in water and 0.01 ml of 1 M-HCl. After 1 min of reaction at 0°C , the solution was neutralized by the addition of 0.025 ml of 1 M- NaHCO_3 in water. Only freshly prepared diazonium solutions were used for the labelling of proteins.

Determination of aromatic amines

The amino group was converted into the diazonium group by the addition of 0.02 ml of 1 M- NaNO_2 in water and 0.02 ml of 1 M-HCl to a sample of 0.20 ml. After 1 min at 0°C , 2 ml of a β -naphthol solution with the following composition was added: 250 μmol of β -naphthol dissolved in 200 ml of ethanol/water/1 M- NaHCO_3 (3:3:2, by vol.). The A_{490} was measured.

Labelling of lactate dehydrogenase, isoenzyme M_4 , and bovine serum albumin with DD ^{125}IBS

Crystals of lactate dehydrogenase (5 mg) were collected by centrifugation for 30 min at 2°C and 18000 g. They were dissolved in 1 ml of 0.1 M-sodium pyrophosphate/HCl buffer (pH 8.1). The solution was dialysed against four changes of 500 ml

of the same buffer for 48 h. The lactate dehydrogenase solution was centrifuged and the clear supernatant was used for further experiments. To 4 vol. of lactate dehydrogenase solution was added 1 vol. of diazonium solution. A 10-fold molar excess of lactate dehydrogenase over DD ^{125}IBS was used. After 2 h of reaction at 0°C , the reaction was stopped by the addition of NaN_3 in 0.08 M-sodium pyrophosphate/HCl buffer (pH 8.1) to a final concentration of 0.02%. Next, the solution of labelled lactate dehydrogenase was dialysed against four changes of 500 ml of 0.1 M-sodium phosphate buffer (pH 7.0) (the first change in the presence of 0.02% NaN_3) at 2°C for 48 h, after which the protein solution was loaded onto a 5'-AMP-Sephacrose 4B column (1 cm $\times$ 5 cm) to purify the labelled lactate dehydrogenase. The column was washed with 25 ml of 0.1 M-sodium phosphate buffer (pH 7.0) and, next, the DD ^{125}IBS -labelled lactate dehydrogenase was eluted with 25 ml of 2 mM-NADH in the same buffer. Fractions (1 ml) were collected and aliquots were analysed for radioactivity and enzyme activity. Fractions containing the radiolabelled lactate dehydrogenase were pooled and dialysed against four changes of 500 ml of phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate, pH 7.4). The dialysed solution was centrifuged for further experiments (30 min at 18000 g). The loss of enzyme activity during the labelling and purification procedure was less than 15%.

Bovine serum albumin was labelled with DD ^{125}IBS in the same way as described for lactate dehydrogenase. The labelled protein was purified by screening. DD ^{125}IBS -labelled bovine serum albumin in phosphate buffered saline (1 ml, 1 mg/ml) was injected into a rat and blood was collected after 1 h. The serum was used for the clearance studies.

More than 95% of the radioactivity of labelled lactate dehydrogenase and bovine serum albumin was acid-precipitable. The preparations contained approx. 1 molecule of DD ^{125}IBS per 100 molecules of protein.

Labelling of bovine serum albumin with ^{125}I

^{125}I -labelled bovine serum albumin was prepared by the Chloramine-T procedure as described by Bocci (1969). Unreacted iodide ions were removed by dialysis against four changes of about 2 litres of phosphate-buffered saline at 2°C . The first change also contained 0.1% (w/v) NaI. The ^{125}I -labelled bovine serum albumin was purified by screening as described above for DD ^{125}IBS -labelled bovine serum albumin.

Handling of animals and liver fractionation

Male rats of an inbred Wistar strain, body wt. 150–250 g, were used. Anaesthesia was induced and maintained with Fluothane in a mixture of nitrous

oxide and oxygen. Rats were injected via the vena penis with 0.2 ml of solution per 100 g of rat. The injected solution contained, for lactate dehydrogenase isoenzyme M_4 , 1 mg of protein/ml of phosphate-buffered saline. Blood samples of about 0.15 ml were obtained from the orbital plexus and centrifuged in heparinized tubes. Triplicate samples of plasma were used for radioactivity measurements and/or enzyme determinations.

Livers were perfused with phosphate-buffered saline at room temperature until they became yellowish. Spleens were not perfused. Spleens and livers were homogenized in 5 and 35 ml of an ice-cold 0.25 M-sucrose solution respectively.

In our calculations, we have used the following relationship between the plasma volume and the body weight:

$$\text{Plasma volume (ml)} = (0.0291 \times \text{body weight in g}) + 2.54$$

(Bijsterbosch *et al.*, 1981). Uptake in spleen and perfused liver was corrected for the appropriate amount of radioactivity in residual plasma present in the tissue at the time of sampling. A value for spleen (85 μ l of plasma/g fresh wt.) was taken from Everett *et al.* (1956). A value of 35 μ l of plasma/g fresh wt. was taken for the amount of plasma left behind after perfusion of liver with phosphate-buffered saline (Munniksmas *et al.*, 1980).

Liver fractionations were carried out as described by Bouma & Gruber (1966).

Determination of protein and enzymes

Protein concentrations were determined by the Lowry method with bovine serum albumin as standard. Acid phosphatase was measured by the method of Gianetto & de Duve (1955) with 2-glycerophosphate as substrate. Glucose-6-phosphatase and acid deoxyribonuclease were determined as described by de Duve *et al.* (1955). Lactate dehydrogenase was determined as described by Sinke *et al.* (1979).

Assay of radioactivity

Radioactivity was measured in a Nuclear-Chicago mark I liquid-scintillation counter. ^{125}I -

containing samples were counted as gamma emitters in a heterogeneous system using tetrabutyltin-loaded scintillator (Gamma-vial C, Koch-Light). The counting efficiency was 45–50%.

Results and discussion

Half-life studies

Derivatization with DD ^{125}I BS did not alter the clearance of lactate dehydrogenase isoenzyme M_4 (Table 1). Acid-precipitable radioactivity and enzyme activity disappeared with the same rate from the blood stream. The rate was not significantly different from that found by Sinke *et al.* (1979) for unlabelled and ^{125}I -labelled lactate dehydrogenase isoenzyme M_4 (Table 1). The clearance followed first-order kinetics. Extrapolation to zero time gave a plasma concentration corresponding to the injected dose divided by the plasma volume. Our labelled preparation therefore behaved as a homogeneous compound.

In experiments with bovine serum albumin one preparation was labelled with ^{125}I and another with DD ^{125}I BS. After a relatively rapid phase, probably due to distribution over the extravascular space, clearance followed first-order kinetics and there was no significant difference between the circulation half-lives of the two preparations (Table 1). The values agree with those found by Van Zile *et al.* (1979) for [^3H]raffinialdehyde-labelled and ^{125}I -labelled bovine serum albumin (23.5 h).

Uptake of DD ^{125}I BS-labelled lactate dehydrogenase isoenzyme M_4 by liver and spleen of rats and fate of breakdown products

To test the new label for its behaviour after uptake of the protein, we determined total and trichloroacetic acid-soluble radioactivity in liver and spleen after injection of DD ^{125}I BS-labelled lactate dehydrogenase isoenzyme M_4 . Previously, Sinke *et al.* (1979) found with ^{125}I -labelled lactate dehydrogenase isoenzyme M_4 that radioactivity in liver and spleen increased up to maxima of about 9 and 3% of the injected dose respectively 13 min after injection

Table 1. Half-lives of intravenously injected lactate dehydrogenase isoenzyme M_4 and bovine serum albumin. Clearance was followed for at least three half-lives and at least four samples per animal were taken for the calculation of the half-lives. Values for half-life are means $\pm$ S.E.M. for the number of experiments given in parentheses.

Protein	Assayed by:	Half-life
DD ^{125}I BS-labelled lactate dehydrogenase isoenzyme M_4	Enzyme activity	29.0 $\pm$ 2.6 min (9)
DD ^{125}I BS-labelled lactate dehydrogenase isoenzyme M_4	Radioactivity	26.3 $\pm$ 1.2 min (9)
Unlabelled lactate dehydrogenase isoenzyme M_4	Enzyme activity	27.7 $\pm$ 1.7 min (4)*
^{125}I -labelled lactate dehydrogenase isoenzyme M_4	Enzyme activity	32.0 $\pm$ 2.6 min (4)*
^{125}I -labelled lactate dehydrogenase isoenzyme M_4	Radioactivity	32.4 $\pm$ 1.6 min (4)*
DD ^{125}I BS-labelled bovine serum albumin	Radioactivity	25.8 $\pm$ 1.5 h (5)
^{125}I -labelled bovine serum albumin	Radioactivity	21.6 $\pm$ 0.4 h (5)

* Values have been taken from Sinke *et al.* (1979).

Fig. 2. Uptake and breakdown of DD¹²⁵IBS-labelled lactate dehydrogenase isoenzyme M₄ by liver

Rats were intravenously injected with about 0.4 mg of protein (2 μ Ci of ¹²⁵I). At the times indicated, livers were perfused with saline and total (●) and 10% (w/v) trichloroacetic acid-soluble (○) radioactivity were counted. Each point represents the mean value for at least three animals. Vertical bars give the S.D.

Fig. 3. Uptake and breakdown of DD¹²⁵IBS-labelled lactate dehydrogenase, isoenzyme M₄, by spleen

Rats were intravenously injected with about 0.4 mg of protein (2 μ Ci of ¹²⁵I). At the times indicated, total (●) and 10% (w/v) trichloroacetic acid-soluble (○) radioactivity were counted. Each point represents the mean value for at least three animals. Vertical bars give the S.D.

and subsequently rapidly declined; 2 h after injection, less than 1% of the injected dose was present in the tissues. Now, gratifyingly, we found with the label DD¹²⁵IBS that radioactivity in liver (Fig. 2) and spleen (Fig. 3) increased up to about 2 h after injection (2 h after injection lactate dehydrogenase isoenzyme M₄ has been almost completely cleared from the blood) and subsequently declined with a half-life of approx. 20 h in both tissues. A significant fraction of the DD¹²⁵IBS-labelled degradation products was precipitated by 10% (w/v) trichloroacetic acid, even at 24 h after injection. This suggests that DD¹²⁵IBS may sterically inhibit the action of proteinases, leaving some undegraded peptides attached to the radioactive label. The same phenomenon was found by Van Zile *et al.* (1979) using [³H]raffinalsedehyde as label and by Pittman *et al.* (1979a,b) using [¹⁴C]sucrose-dichlorotriazine as label.

Up to 48 h after injection, no trichloroacetic acid-soluble radioactivity could be detected in the plasma. Significant amounts of radioactivity were

found in urine (36% of the injected dose) and in faeces (15%) 24 h after injection.

The half-life of disappearance of radioactivity from the tissue (about 20 h) is lower than the value of >100 h with the labels [³H]raffinalsedehyde and [¹⁴C]sucrose-dichlorotriazine. Two possible explanations can be given for this difference. 1. In the lysosomes, the ester linkage may be split by lysosomal esterases giving rise to a compound which diffuses more easily through the membrane. 2. Our label is more hydrophobic than the others and therefore should pass the lysosomal membrane more rapidly.

The contribution of liver and spleen towards the clearance of lactate dehydrogenase, isoenzyme M₄, can be calculated from its half-life in plasma and its uptake by the organs within the period that no loss of radioactively labelled breakdown products into the circulation occurs (Sinke *et al.*, 1979). Using ¹²⁵I as label, Sinke *et al.* (1979) calculated that about 65% and 12% of the injected dose were taken up and

Table 2. Uptake of lactate dehydrogenase isoenzyme M_4 by liver and spleen of rats
Values are means $\pm$ S.E.M.

Label	Uptake (% of dose)			
	Liver		Spleen	
	Maximal experimentally found	Calculated	Maximal experimentally found	Calculated
$^{125}\text{I}^*$	9.1 ± 0.6	41.4 ± 3.1	3.0 ± 0.2	11.0 ± 1.1
DD ^{125}IBS	38.3 ± 0.9	41.0 ± 2.0	10.1 ± 0.8	10.2 ± 0.7

* The results with ^{125}I -labelled lactate dehydrogenase isoenzyme M_4 have been taken from Sinke *et al.* (1979).

Fig. 4. Distribution of acid phosphatase, acid deoxyribonuclease, glucose-6-phosphatase, and total, acid-precipitable and acid-soluble radioactivity over subcellular fractions of rat liver after intravenous injection of DD ^{125}IBS -labelled lactate dehydrogenase, isoenzyme M_4 .

Livers were homogenized and fractionated by differential centrifugation 2 h after injection of the protein. Blocks from left to right represent fractions in the order in which they were isolated: nuclear, mitochondrial, lysosomal, microsomal, and final supernatant fractions. The relative protein content is given on the abscissa. The ordinate represents mean relative specific activity (percentage of total recovered activity divided by percentage of total recovered protein). Thus, the area of each block is proportional to the percentage of activity per fraction. Results given are means of two experiments. Average recoveries from the homogenates were: acid phosphatase, 94%; acid deoxyribonuclease, 104%; glucose-6-phosphatase, 94%; total radioactivity, 104%; acid-precipitable radioactivity, 97%; acid-soluble radioactivity, 95%; and protein, 98%.

subsequently broken down by liver and spleen respectively. However, they did not make corrections for the amount of plasma left behind in the liver after perfusion with saline and they used a too low value for the residual plasma present in the spleen at the time of sampling. We have recalculated the contributions of liver and spleen and found values of 41% and 11% of the injected dose respectively.

These values agree with the experimentally found maximal uptake of 38% and 10% of the injected dose by liver and spleen respectively using DD ^{125}IBS as label (Table 2).

Subcellular distribution of radioactivity

The subcellular localization of DD ^{125}IBS -labelled lactate dehydrogenase isoenzyme M_4 in liver was

studied by differential centrifugation 2h after injection of the protein. Fig. 4 shows the distribution of total radioactivity, acid-precipitable radioactivity and acid-soluble radioactivity, of the lysosomal marker enzymes acid phosphatase and acid deoxyribonuclease, and of the microsomal marker enzyme glucose-6-phosphatase. About 70% of the radioactivity is present in particles having a range of sedimentation constants that spans those of mitochondria and lysosomes. About 20% of the radioactivity (both acid-soluble and acid-precipitable) is found in the final supernatant, probably representing disrupted lysosomes. The distribution patterns of total, acid-soluble and acid-precipitable radioactivity are similar, indicating that no acid-soluble radioactivity has accumulated in the extralysosomal compartment of the cell during the first 2h. The distribution of radioactivity resembles that of acid deoxyribonuclease [which is concentrated in lysosomes of sinusoidal cells (Munthe-Kaas *et al.*, 1976)] rather than that of acid phosphatase [which occurs mainly in lysosomes of hepatocytes (Munthe-Kaas *et al.*, 1976)], indicating that lactate dehydrogenase, isoenzyme M₄, is mainly or only taken up by sinusoidal cells.

Conclusions

From the experiments presented above it is clear that DD¹²⁵IBS as label is clearly superior to ¹²⁵I. The new label does not seem to influence blood clearance or uptake in liver and spleen. Following protein removal from the circulation and intracellular degradation in lysosomes, the DD¹²⁵IBS-labelled degradation products disappear very slowly (*t*_{1/2} is approx. 20h) from liver and spleen, in contrast to radioactivity from ¹²⁵I-labelled proteins which is rapidly lost from tissues (*t*_{1/2} < 30min). The new radioactive tracer seems promising to identify sites of catabolism of plasma proteins with relatively long circulating half-lives.

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