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A radioiodinated, intracellularly trapped ligand for determining the sites of plasma protein degradation *in vivo*

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We recently developed a general method for determining tissue sites of degradation of plasma proteins *in vivo* that made use of covalently attached radioactive sucrose. On degradation of the protein, the sucrose remained trapped in the cells as a cumulative marker of protein degradation. The method described here depends on the same principles, but uses an adduct of cellobiose and tyramine that is radioiodinated to high specific radioactivity and then covalently attached to protein. Use of the radioiodinated ligand increases the sensitivity of the method at least 100-fold and allows simplified tissue analysis. Proteins derivatized with the radioiodinated ligand were recognized as underivatized proteins both *in vitro* and *in vivo*. On degradation of derivatized low-density lipoprotein, the rate of leakage from cultured fibroblasts was only 5% during 24h. Similarly, on injection of labelled proteins into rats and rabbits, urinary excretion of the label was in all cases less than 10% of total labelled catabolic products recovered 24h after injection. Examination of the tissue contents of label at two times after injection of labelled asialofetuin or apolipoprotein A1 in rats, and asialotransferrin in rabbits showed that the label did not detectably redistribute between tissues after initial uptake and catabolism; a significant leakage from liver was quantitatively accounted for by label appearing in gut contents and faeces. A simple double-label method was devised to provide a correction for intact protein in trapped plasma, the extravascular spaces, and within cells. By using this method it becomes unnecessary to fractionate tissue samples.

Until recently there was no general method available for accurately quantifying the tissue sites of degradation of plasma proteins. Proteins labelled by conventional methods, most importantly by direct radioiodination, yield radiolabelled catabolic products that rapidly escape the cells for excretion as iodide or iodotyrosine. Consequently the label residual in the cells at any time need bear no direct relationship to the ongoing rate of catabolism by those cells.

We have described a general approach to this problem (Pittman & Steinberg, 1978; Pittman *et al.*, 1979*a*). A similar approach was described by Van Zile *et al.* (1979). We covalently attached [^{14}C]-sucrose to proteins by using cyanuric chloride as a linking agent. The [^{14}C]sucrose moiety did not alter the metabolism of the labelled protein, and indeed was well trapped intracellularly on degradation of the protein. The small amount of [^{14}C]sucrose-labelled catabolic products that did escape from cells did not redistribute to other tissues (Pittman *et al.*, 1979*a*, 1982*a*). Thus the ^{14}C accumulating within a tissue was an integrated measure of [^{14}C]sucrose-labelled protein that had been catabolized by that tissue. It can be shown that, in any case in which the labelled protein is introduced into the same compartment where newly synthesized tracee protein enters, the accumulation of label at very long times after injection is in proportion to the mass of protein degraded (Pittman *et al.*, 1979*b*; Carew & Beltz, 1982). Thus the label distribution is in proportion to their contributions to overall protein degradation.

However, the sucrose-labelling method is limited by the maximum specific radioactivities that can be obtained. It would be highly desirable to have available an intracellularly trapped label analogous

Abbreviations used: LD lipoprotein, low-density lipoprotein; TC, tyramine–cellobiose adduct; [*I-TC]-protein, protein labelled by covalent attachment of the radioiodinated TC ligand; [*I-TC],*I-protein, protein labelled both by attachment of the TC ligand and by direct iodination; apo A1, apolipoprotein A1.

to [^{14}C]sucrose but incorporating radioiodine at high specific radioactivity. This would allow more precise assay of tissues of low degradative capacity, and the ability to trace the fates of proteins that are catabolized slowly or proteins present at very low concentrations in plasma. DeJong *et al.* (1981, 1982) prepared and used an intracellularly trapped label that made use of a radioiodinated derivative of sucrose. However, because of a high rate of leakage of label to urine and faeces, use of the label would be restricted mainly to rapidly catabolized proteins.

In the present paper we describe the preparation and use of a radioiodinated trapped label, an adduct of tyramine and cellobiose attached to protein using cyanuric chloride, which is analogous to the sucrose label previously described. We show that proteins derivatized with this label are recognized as underivatized proteins by biological systems, and that the label indeed is adequately trapped intracellularly after uptake and catabolism of the protein. We further show than an internal-correction method using conventional radio-iodination to trace undegraded protein is useful in correcting the tissue content of trapped label for the contribution of undegraded protein.

Materials and methods

Synthesis of the tyramine–cellobiose adduct

Tyramine was linked to cellobiose by reductive amination with sodium cyanoborohydride ($NaBH_3CN$) to reduce the transient Schiff base, giving a stable carbon–nitrogen bond that is not amenable to hydrolysis in cells. Cellobiose, tyramine and $NaBH_3CN$ were obtained from Sigma Chemical Co. The three reagents were solubilized at equimolar concentrations (0.12M) in 0.2M-sodium phosphate buffer (4.55g of cellobiose, 1.82g of tyramine and 0.83g of $NaBH_3CN$ in 100ml at pH7.5). Tracer [^{14}C]tyramine (100μCi) was added to facilitate later quantification and identification of products. The mixture was allowed to react for 6 days at room temperature. It was then adjusted to pH5.5 with HCl and applied to a column (2cm × 20cm) containing a strongly acidic cation-exchange resin (Bio-Rad AG-50W; H^+ form). The column was eluted with water (300–400ml) and then with 0.5M-NH_3. The material eluting with NH_3, which contained the adduct and unchanged tyramine, was freeze-dried. The adduct and free tyramine were resolved by silicic acid chromatography on a 2cm × 25cm column eluted with butanol/acetic acid/water (7:1:2, by vol.). Tyramine preceded the product from the column, being eluted at about 50ml. The major product was eluted over the next 70ml. It was analysed by t.l.c. on silica gel, with butanol/acetic acid/water (7:1:2, by vol.) as the developing solvent. The major product stained for both carbohydrate (Partridge, 1948) and amine (Greig & Leaback, 1960) moieties. This product partially overlapped a by-product also containing both carbohydrate and amine moieties that was eluted beginning at about 90ml. The region containing the major product, but excluding the more retained by-product, was pooled and freeze-dried. Further purification was achieved by gel filtration on Sephadex G-10, which removed a lower-molecular-weight component. The final product displayed one predominant band on t.l.c. with a small contaminant of slightly slower migration.

To quantify the purity of the product, the two components were resolved by paper chromatography using the developing system described above and extracted from the paper. The u.v. spectra of these components were indistinguishable from each other and from that of free tyramine. Measurement of absorbance at its maximum (279nm) disclosed 83% major component and 17% minor component, assuming equal absorption coefficients. The impurity is thought, on the basis of its staining behaviour with carbohydrate-specific and amine-specific reagents, to be the product of reaction of two molecules of cellobiose with one of tyramine. The amine group of tyramine in such a complex would not be available for further reaction. In some studies the product was prepared free of the minor component by preparative t.l.c. in the system outlined above and used in labelling studies. Alternatively, the product from silicic acid chromatography was purified to yield a single component by column chromatography on silica gel, being eluted with chloroform/methanol/water/conc. NH_3 (sp.gr. 0.88) (64:31:2:3, by vol.). The behaviour of the fully purified preparation and the less pure preparation were indistinguishable. The 83%-pure preparation was stored at −6°C and used for most of the studies described here.

Radioiodination of the tyramine–cellobiose adduct and attachment to protein

The tyramine–cellobiose adduct was radioiodinated before its activation and binding to protein. Iodination was performed with carrier-free radioiodide and 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril, a water-insoluble oxidizing agent (Iodogen; Pierce Chemical Co.) (Fraker & Speck, 1978). In the usual procedure a microreaction vessel was coated with 10μg of Iodogen introduced in methylene chloride, which was gradually evaporated by hand-warming. The coated vial was washed vigorously with water several times to remove any lightly adhering material. To the Iodogen-coated vessel was added 30–100nmol of the tyramine–cellobiose adduct in 3–10μl of 0.02M-sodium phosphate buffer, pH7.2. The desired amount of radioactive NaI (usually 2–5mCi) was added and reaction was

allowed to proceed for about 30min at room temperature. After this treatment, the mixture was transferred to a fresh vial containing 10μl of 0.1M-sodium bisulphite and 5μl of 0.1M-NaI to stop further reaction. The transfer was made taking care not to touch the coated wall of the vial. The efficiency of iodination was generally more than 95%. This was determined by removing a trace amount of the reaction mixture to a tube containing unlabelled NaI in 5% trichloroacetic acid. H_2O_2 was added to oxidize iodide to iodine, which was quickly extracted into chloroform by three extractions. Radioiodine remaining in the aqueous phase was taken as a measure of adduct-bound radioiodine. The radioiodinated tyramine–cellobiose adduct was used without separation from residual radioiodide, NaI and sodium bisulphite.

The radioiodinated adduct was allowed to react with a cross-linking agent, cyanuric chloride, for binding to protein. To the 35–75μl of radioiodinated adduct was added 1mol.-equiv. (30–100nmol) of cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) in 20μl of acetone, and 2mol.-equiv. (60–200nmol) of NaOH in 5μl of water. (The cyanuric chloride had been purified by two recrystallizations from toluene.) After 10–30s, 3mol.-equiv. (90–300nmol) of acetic acid in 5–20μl of water was added to quench further reaction. The resulting activated ligand was used immediately for binding to protein, although reactivity was maintained for at least 1h.

The active group of TC that binds to cyanuric chloride was not conclusively identified, but several lines of evidence support the conclusion that it is the secondary amine linking the sugar and triazine residues. First, it was shown that the sugar moiety is unlikely to be involved in this binding; model carbohydrates, including cellobiose and sorbitol, did not significantly compete with radioiodinated TC for activation and binding to protein, even at concentrations up to 50 times the molar concentration of TC ligand (100 times the concentration of cyanuric chloride in this case). Furthermore, in previous studies in which [^{14}C]sucrose was bound to protein via cyanuric chloride, binding of rather low efficiency was achieved and only at pH more than 11 compared with pH8.0–8.5 in the present studies, and only with a 1mol.-equiv. excess of cyanuric chloride over [^{14}C]sucrose. Second, in the present studies, adding excess cyanuric chloride did not increase binding of radioiodinated TC to protein, and decreasing the ratio of cyanuric chloride to TC to below 1 produced a proportionate decrease in binding. This suggests that one TC molecule, and only one, reacts with each cyanuric chloride molecule. Finally, a model secondary amine, diethylamine, competed with iodinated TC for activation by cyanuric chloride and binding to protein as effectively as non-radioactive TC itself and more effectively than a model phenolic compound, *p*-cresol. This suggests that the secondary amine of TC is the more reactive nucleophile under the normal conditions of the activation reaction. However, unlabelled TC did not compete for binding of radioiodinated TC as efficiently as would be expected if both species were equally reactive, leaving open the possibility that the radioiodination might favour reaction via the phenolic hydroxy group.

For all proteins except LD lipoprotein, the activated ligand was added to an appropriate amount of protein, which was at a concentration of 2–10mg/ml in 0.1M-sodium phosphate buffer, pH7.0–7.5. In these cases, about 0.8mol of ligand was added per mol of protein in order to obtain a product with no significant population of molecules carrying more than one ligand. Reaction was allowed to proceed for at least 1h, at which time binding was nearly maximal. In the case of LD lipoprotein, 1μmol of ligand was allowed to react per 100mg of LD-lipoprotein protein. The reaction was carried out for 20–30min at pH9.0–9.5. In all cases the labelled protein was made to 0.1M with respect to NH_4HCO_3 and either dialysed against 0.1M-NH_4HCO_3 or applied to appropriate gel-filtration columns and eluted with 0.1M-NH_4HCO_3. (Columns were chosen to just exclude the protein.) At least for LD lipoprotein this exposure to NH_4HCO_3 was important in removing a reversibly bound fraction of the TC label. Exhaustive dialysis was then carried out against 0.154M-NaCl buffered with 0.02M-sodium phosphate, pH7.4, and containing 0.1% EDTA. The efficiency of ligand binding to protein was generally more than 70%. This was determined in terms of the amount of radioiodine bound to protein, corrected for that fraction of radioactivity present as unbound radioiodide. The extent of derivatization was always less than one TC residue per protein molecule, or, in the case of LD lipoprotein, less than one residue per 100kDa of LD lipoprotein. Specific radioactivities at the time of use of the preparations were generally 200–500c.p.m./ng of protein.

All final preparations of labelled proteins except LD lipoprotein contained less than 1% of radioactive iodine soluble in 10% (w/v) trichloroacetic acid. LD lipoprotein contained less than 2%, but less than 5% of label was soluble in 50% (w/v) tetramethylurea, a reagent that delipidates and specifically precipitates the apo B component of LD lipoprotein (Kane, 1973).

Labelling of non-lipoprotein proteins with the TC ligand was straightforward, and no important problems were encountered. Lipoproteins, however, were somewhat more difficult to handle. In previous studies it was found that some preparations of [*I-TC]-LD lipoprotein displayed a lower rate of uptake by cultured fibroblasts and of clearance from

the plasma *in vivo* than did directly iodinated *I-LD lipoprotein. The discrepancy was traced to a labelled component that did not enter the cells with LD lipoprotein, apparently due to exchange on to other longer-lived proteins. In fact most of this component could be removed from LD lipoprotein by incubation with lipoprotein-deficient serum or with albumin alone to yield a preparation of [*I-TC]-LD lipoprotein that then was not distinguished from *I-LD lipoprotein either *in vitro* or *in vivo*. The component co-fractionated with LD lipoprotein on gel filtration, co-precipitated on acid treatment, and was poorly extracted into common lipid solvents. Only precipitation of the apo B component of LD lipoprotein with 50% tetramethylurea allowed its assessment. Although unidentified, it is thought to be the product of *I-TC attachment to phospholipid constituents of LD lipoprotein. Conditions were found that decreased the label soluble in 50% tetramethylurea to less than 5% of total. This involved a higher pH and shorter reaction time for binding of the ligand to LD lipoprotein than was used for other proteins. Higher temperature (37°C) also decreased the amount of the component, but was not used routinely. A similar problem appears to occur when labelling high-density lipoprotein. It will therefore be necessary to test for such a component in all labelled lipoprotein preparations and to determine if the conditions optimal for labelling LD lipoprotein are appropriate for other lipoproteins.

Preliminary evidence indicated that the TC ligand binds to lysine residues. This was studied by using rabbit albumin labelled with the TC ligand. The derivatized protein was exhaustively hydrolysed with Pronase before separation of the glycopeptides by gel filtration and then by affinity chromatography on a gel containing boronic acid residues, which bind vicinal hydroxy groups (Affi-Gel 601; Bio-Rad). Examination of this product by t.l.c. and amino acid analysis indicated that lysine residues were the predominant sites of attachments.

Direct radioiodination of proteins

Conventionally radioiodinated proteins were prepared by using carrier-free radioiodide and Iodogen as oxidizing agent, generally as outlined above for labelling the TC adduct. In some cases proteins were both directly iodinated and also labelled by attachment of the TC ligand. In these cases direct iodination was carried out first to avoid labelling the tyramine moiety with both iodine isotopes. About 5 mg of protein was allowed to react with $Na^{131}I$ or $Na^{125}I$ (usually 1–3 mCi) in a tube coated with 10 μg of Iodogen at pH 7.0–7.5 (for LD lipoprotein, pH 9.5) for less than 30 min (less than 10 min for LD lipoprotein) at room temperature. Efficiency of iodination was generally 80–95%, as determined by precipitation of a small sample in carrier protein with 10% trichloroacetic acid. If necessary, the iodination reaction was continued in a second vial coated with Iodogen. Labelling of the lipid component of LD lipoprotein was about 3%. It was found that LD lipoprotein labelled in this manner was not distinguishable from LD lipoprotein labelled by the ICl method (McFarlane, 1958), either in competition experiments in cultured human fibroblasts or in terms of plasma decay curves in rabbits.

Protein preparations

Human LD lipoprotein, (*d* 1.02–1.06) was isolated by sequential ultracentrifugation, NaBr being used to adjust solution density (Havel *et al.*, 1955). Rat apo A1 was prepared from high-density lipoprotein (*d* 1.12–1.21) as reported in detail elsewhere (Glass *et al.*, 1983). Fetuin and transferrin were purchased from Sigma Chemical Co. and desialylated by using neuraminidase immobilized on agarose beads as previously described (Attie *et al.*, 1980).

Experiments in vivo

Indwelling catheters were placed in the external jugular veins of experimental rats and rabbits under general anaesthesia the day before beginning experimental protocols. Catheters were used for injection of labelled proteins and for withdrawal of blood samples to determine plasma decay kinetics. The kinetics were determined in terms of trichloroacetic acid-precipitable label present in plasma during the time course. At termination of the experiments, animals were killed and tissues taken for radioassay as described previously (Pittman *et al.*, 1982*a*,*b*). In most experiments tissue pieces were placed directly into tubes for radioassay. Samples of about 1 g were used, compressed in the bottoms of the tubes. Where indicated in the Results section, radioiodine was measured in tissue homogenates, generally 1:5 (w/v) in water. Protein-bound radioiodine in these homogenates was determined after precipitation with 10% trichloroacetic acid.

To prevent re-utilization of radioiodide in those rabbits in which conventionally labelled or doubly labelled LD lipoprotein was studied, they were injected with 3 mg of NaI just before injection of the labelled protein.

In all cases when determining both ^{125}I and ^{131}I, appropriate window settings of the γ-scintillation counter and correction factors were used to separate decay spectra of the two isotopes.

Plasma decay data were analysed as previously described (Carew & Beltz, 1982). All curves were adequately fitted, with computer aid, to bi-exponential functions. Fractional catabolic rates were determined as described by Matthews (1957).

Experiments with cultured cells

Studies of LD lipoprotein catabolism in cultured human fibroblasts were carried out as previously described (Pittman *et al.*, 1979*a*; Stein *et al.*, 1976). Cells were incubated in lipoprotein-deficient serum for 24h before addition of labelled lipoprotein. Degradation of both conventionally iodinated proteins and those labelled with the TC ligand was calculated as the sum of total cell-associated radioactivity after trypsin treatment of the cells plus that appearing in the medium as radioiodide soluble in 10% trichloroacetic acid.

Results

There are two important criteria in justifying the use of an intracellularly trapped protein label: the presence of the ligand must not alter the recognition or handling of the protein by biological systems; once incorporated into cells, the labelled degradation products must be adequately trapped. Both of these criteria have been examined with the tyramine–cellobiose label *in vitro* and *in vivo*.

As Fig. 1 shows, derivatization of human LD lipoprotein with the TC ligand did not alter its recognition and uptake by cultured fibroblasts, and the ligand was well trapped on degradation of the protein. This experiment compared the metabolism of TC-labelled LD lipoprotein ([^{125}I-TC]-LD lipoprotein) and directly iodinated LD lipoprotein (^{131}I-LD lipoprotein) by cultured human fibroblasts during exposure to the labelled LD lipoprotein species for 24h. Under these conditions LD-lipoprotein catabolism is a predominantly receptor-mediated process at the lower concentrations studied (Goldstein & Brown, 1977). Radioactivity in catabolic products from ^{131}I-LD lipoprotein was found predominantly in the medium (94%) (Fig. 1*a*). Label from [^{125}I-TC]-LD lipoprotein remained predominantly in the cells, with only 6% escaping to the medium as trichloroacetic acid-soluble products (Fig. 1*b*). Total uptakes of [^{125}I-TC]-LD lipoprotein and ^{131}I-LD lipoprotein were the same (Fig. 1*c*). In five experiments in which tetramethylurea-soluble label was less than 5% of total label (see the Materials and methods section), apparent leakage from fibroblasts of ^{125}I-labelled catabolic products soluble in 10% trichloroacetic acid was 5.0 ± 2.0% of total [^{125}I-TC]-LD-lipoprotein uptake during a 24h period in which cells were exposed to 5–50μg of LD lipoprotein protein per ml (note that all errors in the present paper are expressed as S.D.). The ^{125}I in the cells was generally more than 60% soluble in trichloroacetic acid.

In the experiment shown in Fig. 2, LD lipoprotein was derivatized to various extents with the TC ligand, and its ability to compete with conventionally labelled ^{125}I-LD lipoprotein for uptake and

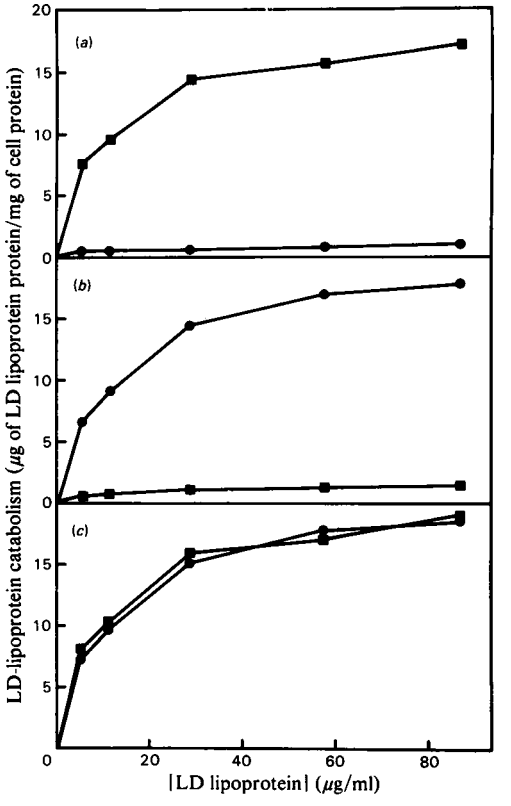

Fig. 1. *Catabolism of [^{125}I-TC]-LD lipoprotein and directly iodinated ^{131}I-LD lipoprotein by cultured human skin fibroblasts*

Cells were incubated with lipoprotein-deficient serum for 24h before addition of the indicated concentrations of LD lipoprotein. [^{125}I-TC]-LD lipoprotein and ^{131}I-LD lipoprotein were mixed with unlabelled LD lipoprotein (more than 80% unlabelled) and applied to the cells at the indicated total concentrations of LD lipoprotein. Cells were harvested 24h later and catabolism of LD lipoprotein determined as outlined in the Materials and methods section. Data were calculated by assuming that each isotope traced total LD lipoprotein. Each point is the mean result for triplicate plates. Two other experiments yielded similar results. Symbols used are: ●, ^{125}I from [^{125}I-TC]-LD lipoprotein; ■, ^{131}I from ^{131}I-LD lipoprotein.

catabolism by fibroblasts was compared with that of underivatized LD lipoprotein. The presence of up to 17 residues of TC ligand per 625kDa of LD lipoprotein (625000 being the presumed molecular weight of apo B in LD lipoprotein) did not significantly alter the ability to compete with ^{125}I-LD lipoprotein in this receptor-mediated process. This extent of derivatization represents reaction of about 3% of total lysine residues, a level found by

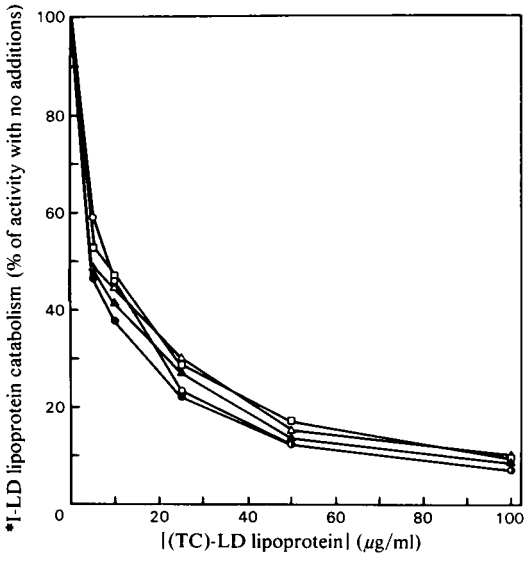

Fig. 2. *Effect of derivatization of LD lipoprotein with the TC ligand on its ability to compete for catabolism of native LD lipoprotein*

LD lipoprotein was derivatized to various extents by using a trace amount of ^{131}I to determine the extent of derivatization. Cultured human fibroblasts were incubated for 24h in lipoprotein-deficient serum before addition of lipoproteins. All plates received conventionally labelled ^{125}I-LD lipoprotein (5μg of protein/ml). The indicated concentrations of unlabelled LD lipoprotein, either underivatized or derivatized to various extents, were added to compete for catabolism of the ^{125}I-LD lipoprotein. Catabolism was assayed 24h later in terms of ^{125}I in the medium soluble in 10% trichloroacetic acid. Results are shown as the percentage of ^{125}I-LD lipoprotein catabolism relative to that in the absence of competing LD lipoprotein. The extents of derivatization of the competing preparations, in terms of TC residues per 625kDa of protein, were: ○, none; ●, 1.0 residue; ▲, 4.5 residues; □, 8.7 residues; △, 17.2 residues. Each point is the mean result for triplicate plates.

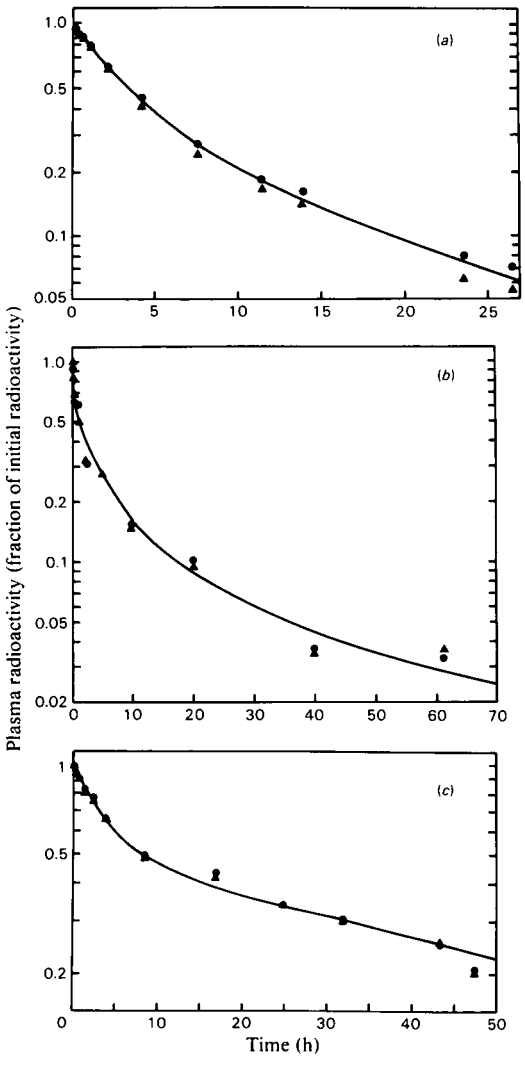

Fig. 3. *Comparison of the plasma decays of proteins labelled with the TC ligand and by direct iodination*

Samples of the proteins were labelled either with the ^{125}I-TC ligand or by direct iodination with ^{131}I. The alternatively labelled preparations were simultaneously injected intravenously, and plasma decays were monitored as outlined in the Materials and methods section. (*a*) Human LD lipoprotein in rabbits; (*b*) rat apo A1 in rats; (*c*) rabbit albumin in rabbits. In all cases: ▲, ^{125}I-TC-labelled proteins; ●, proteins conventionally iodinated with ^{131}I.

Weisgraber *et al.* (1978), who used several lysine modifications, not to appreciably influence receptor binding.

Derivatization of proteins with the TC ligand also did not detectably alter their plasma decay *in vivo*. As shown in Fig. 3, human LD lipoprotein derivatized with ^{125}I-TC exhibited plasma decay kinetics similar to those of ^{131}I-LD lipoprotein in rabbits (Fig. 3*a*). Similarly, the decay of ^{125}I-TC-labelled rat apo A1 in rats was indistinguishable from that of ^{131}I-apo A1 (Fig. 3*b*). Finally, ^{125}I-TC-labelled rabbit albumin decayed in rabbits like albumin directly iodinated with ^{131}I (Fig. 3*c*).

Experiments *in vivo* also disclosed an acceptable rate of leakage from tissues. Asialofetuin, a protein targeted for rapid uptake by liver (Ashwell & Morell, 1974), was labelled with the ^{125}I-TC ligand and intravenously injected into rats. Animals were killed

either 1 h ($n = 3$) or 24 h ($n = 3$) later and tissues were examined for ^{125}I content. After 1 h, the plasma was virtually cleared of ^{125}I, with less than 5% of the injected dose remaining; therefore total ^{125}I in tissues was taken as a measure of catabolism. At 1 h, $90 \pm 3\%$ of the injected ^{125}I was found in the liver. At 24 h, the liver still contained $68 \pm 4\%$ of the injected dose; the gut contents and faeces contained $25 \pm 6\%$, accounting for the loss from liver, presumably by biliary excretion. The rest of the tissues contained $5.3 \pm 1.3\%$ of injected label after 1 h and $5.2 \pm 1.1\%$ after 24 h. Only 2.5% of injected ^{125}I appeared in urine over 24 h. Thus there was very little leakage of the TC label from liver except to bile, and ^{125}I initially taken up by the liver did not later redistribute to other tissues.

In studies of apo A1 metabolism reported in detail elsewhere (Glass *et al.*, 1983), [^{125}I-TC]-apo A1 was intravenously injected into rats and the tissues examined for ^{125}I content 24 h ($n = 4$) or 48 h ($n = 4$) later. After 24 h, more than 90% of [^{125}I-TC]-apo A1 had been irreversibly cleared, and again total label content of the tissues was used as a measure of degradation. In this case no single organ predominated in catabolism of the protein. At 24 h, only $6 \pm 3\%$ was recovered in urine. At 48 h label in urine had increased to $9 \pm 2\%$. The only tissue with ^{125}I content significantly different from that at 24 h was liver, where a small decrease in label content was matched by a rise in label in gut contents. Thus there was an acceptably low leakage rate, and again no evidence of label redistribution.

The adequacy of trapping of the *I-TC was also studied by using asialotransferrin, a protein targeted for uptake by both liver parenchymal cells and cells of bone marrow (Regoeczi *et al.*, 1980). Transferrin was desialylated as described in the Materials and methods section, labelled with the ^{125}I-TC ligand, and intravenously injected into four rabbits. After 3 h, the plasma was nearly cleared of ^{125}I (less than 10% of that injected remaining). At that time, tissues from two rabbits were examined for ^{125}I content; the label was found predominantly in liver (50% of recovered ^{125}I) and in bone marrow [27%, assuming that 3.5% of body mass represents bone marrow (Wintrobe, 1967)]. The other two animals were killed 24 h after injection; 40% of recovered ^{125}I was still in the liver and 31% in bone marrow. The 10% decrease in label in liver between 3 and 24 h was accounted for in gut contents and faeces. The contents of other tissues were not significantly different at 3 and 24 h. Although urinary excretion was not directly measured in these animals, it can be concluded that here again there was an acceptably low rate of leakage (assuming label in gut contents and faeces is attributable to initial uptake by liver) and no evidence for redistribution of label to other tissues.

An internal correction method for trapped undegraded TC-labelled protein

In the studies described below, tissues were assayed at times after injection that were long compared with the plasma residence of the proteins, in all cases (except the early asialotransferrin time point) at least four times the half-time for plasma clearance. Assuming a model for catabolism of plasma proteins described by Matthews (1957), well over 90% of the protein had been catabolized in these cases and little label remained on intact protein. However, this would not be true of less rapidly catabolized proteins studied at short times after injection, relative to their plasma clearance rates. In those cases the presence in tissues of label on intact protein in trapped plasma and the intravascular spaces may make an important contribution to total radioactivity that is not in proportion to degradation. Unfortunately, no simple, generally applicable method for physically separating degradation products from intact protein has been found. With both the [^{14}C]sucrose and the *I-TC intracellularly trapped labels, much of the labelled degradation products accumulating in tissues remain attached to short peptides that are in part precipitated by trichloroacetic acid and other common protein precipitants (Pittman *et al.*, 1979*b*, 1982*b*).

We have sought to obviate this problem by using an internal-correction method to correct for label on undegraded protein. Direct iodination of tyrosine moieties was used to trace the intact protein. This approach requires that label from conventionally iodinated protein represent predominantly undegraded protein, and that label in catabolic products of conventionally iodinated protein residual in tissues be small compared with the amount of label from TC-labelled protein trapped in those tissues as catabolic products. This in turn requires that radioiodinated products from catabolism of directly iodinated protein be rapidly cleared from the tissues. These conditions are approximately met at fairly long times after injection of directly radioiodinated proteins, that is, at times long compared with the time required for processing through cells and long compared with the turnover of the body radioiodide pool (Berman, 1972; Matthews, 1957). For example, 24 h after injection of conventionally iodinated LD lipoprotein in pigs, only a small fraction of the injected dose remains in tissues, mainly as undegraded protein (Sniderman *et al.*, 1975; Calvert *et al.*, 1975).

The principle of this internal-correction approach is illustrated by preliminary experiments *in vitro* exemplified in Fig. 4. A rabbit was injected with LD lipoprotein labelled both with the ^{125}I-TC ligand and by direct iodination with ^{131}I. It was killed 24 h later, and an homogenate prepared from the liver. This was subjected to gel filtration on 8% agarose. Of the

total ^{125}I recovered, 89% was retained beyond the void volume as degradation products. In contrast, ^{131}I was found predominantly in the exclusion volume; ^{131}I, therefore, traced mainly intact or nearly intact protein. When ^{131}I in the exclusion volume was multiplied by the $^{125}I/^{131}I$ ratio of the original doubly labelled LD lipoprotein preparation and subtracted from the total ^{125}I recovered from the column, it was calculated that only 9% of the ^{125}I was accounted for in intact or nearly intact LD lipoprotein. Therefore 91% of the ^{125}I was calculated by this method to be in degradation products, in good agreement with the 89% directly determined by retention of ^{125}I on gel filtration. It is noteworthy that much of the ^{125}I retained on 8% agarose was of molecular weight well over 1000, apparently representing the TC ligand still attached to short peptides. Only 66% of these catabolic products retained by the gel column were soluble in 10% trichloroacetic acid.

To test the internal-correction approach *in vivo*, human LD lipoprotein was directly iodinated with ^{131}I and then labelled with the ^{125}I-TC ligand. The resulting doubly labelled LD lipoprotein ([^{125}I-TC],^{131}I-LD lipoprotein) was injected into rabbits. Animals were killed 24h later, and the tissues were examined for total ^{125}I and ^{131}I content and for their content of the two isotopes soluble and precipitable in 10% trichloroacetic acid. An experiment representative of five of this type is shown in Table 1. Values are the averages from two rabbits injected with a single preparation of [^{125}I-TC],^{131}I-LD lipoprotein. Tissues of high, moderate and low specific radioactivities are shown. Activities are expressed as that portion of the plasma fractional catabolic rate attributable to 1g of tissue. These values are calculated as:

$$\frac{^{125}I \text{ in catabolic products from 1 g of tissue}}{\text{Total } ^{125}I\text{-catabolic products recovered}} \times \text{plasma fractional catabolic rate}$$

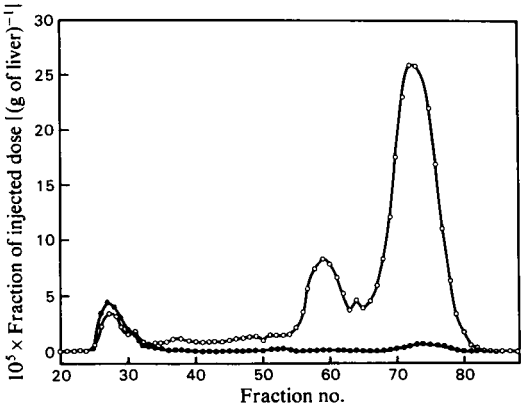

Fig. 4. *Gel-filtration chromatography of a homogenate extract of liver from a rabbit injected with doubly labelled [^{125}I-TC],^{131}I-LD lipoprotein*

A rabbit was killed 24h after injection of the labelled lipoprotein. The liver was homogenized in water (1:5, w/v) and a supernatant fraction was prepared by centrifugation at 20000*g* for 30min (79% recovery of total liver ^{125}I, and 81% recovery of ^{131}I). This fraction was applied (4ml) to a column (1.5cm × 50cm) of 6% agarose eluted with phosphate-buffered saline (0.1M-sodium phosphate/10mM-EDTA/0.9% NaCl, pH 7.4). To normalize the values for the two isotopes, the content of each label in each fraction (corrected for decay) was divided by the injected dose of label, and by the weight of liver represented by the amount of applied liver extract, to give the fraction of the injected dose per g of tissue in each fraction. ○, ^{125}I; ●, ^{131}I.

Table 1 compares two ways of determining the amount of ^{125}I in tissues as catabolic products of [^{125}I-TC],^{131}I-LD lipoprotein. In method 1, pieces of tissue were directly radioassayed for total ^{125}I and ^{131}I content. The ^{125}I content was corrected for the presence of undegraded [^{125}I-TC],^{131}I-LD lipoprotein, assuming all ^{131}I in the tissue was on intact [^{125}I-TC],^{131}I-LD lipoprotein. Thus the tissue's ^{131}I content multiplied by the $^{125}I/^{131}I$ ratio of the doubly labelled preparation was subtracted from the total ^{125}I content. In method 2, it was not assumed that ^{131}I represented only undegraded protein. Rather, ^{131}I precipitable in 10% trichloroacetic acid was used to

Table 1. *Specific activities of selected rabbit tissues in degradation of LD lipoprotein, determined by two methods*

Tissue specific activity	10^3 × Fractional catabolic rate per g wet wt. of tissue ($h^{-1} \cdot g^{-1}$) Method 1	Method 2
High		
Adrenal	1.94	1.82
Liver	0.394	0.370
Spleen	0.906	0.853
Moderate		
Kidney	0.105	0.108
Ovary	0.074	0.073
Intestine	0.020	0.023
Lymph nodes	0.017	0.019
Low		
Muscle	<0	0.002
Skin	<0	0.005
Adipose tissue	0.001	0.003
Thymus	0.001	0.004

correct the tissues' total ^{125}I content for that still on undegraded protein to give a value for ^{125}I present in degradation products.

As Table 1 shows, agreement between the two methods was good for tissues of high and moderate specific radioactivities. However, the least-active tissues were underestimated by the first method. This may be explained in terms of the least-active-tissues' content of ^{131}I that arose from catabolism of [^{125}I-TC],^{131}I-LD lipoprotein in other tissues. Because of the size of the iodide pool and its approximately uniform distribution, all tissues carry a 'background' of $^{131}I^-$. In low-uptake tissues, such as muscle, this may be large compared with the accumulated ^{125}I-labelled catabolic products. These tissues consequently may even display a negative value for ^{125}I content corrected for intact protein by using total ^{131}I content, as was true for the less-than-zero values shown in Table 1. The over-correction occurs only in those tissues that contribute little to total protein degradation. Overall, catabolism of [^{125}I-TC],^{131}I-LD lipoprotein will be underestimated only to the extent that $^{131}I^-$ or other ^{131}I-labelled products from catabolism of the doubly labelled particles remains in tissues. In the experiment of Table 1, the sum of the trichloroacetic acid-soluble ^{131}I recovered in all tissues, corrected for the specific radioactivities of ^{125}I and ^{131}I in the original LD-lipoprotein preparation, was equivalent to only 5% of the total ^{125}I-labelled degradation products recovered in all tissues. Thus, in this experiment, the use of method 1 underestimated the catabolism of [^{125}I-TC],^{131}I-LD lipoprotein overall by only 5% compared with method 2, and underestimation was apparent only in tissues of low catabolic activity. Where careful evaluation of the least-active tissues is needed, the more cumbersome, but precise, method 2 can be used.

Discussion

The intention of the present work was to devise a methodology, based on the principles previously developed for the sucrose trapped label, that would utilize a higher-specific-radioactivity γ-radiation-emitting ligand. This eliminates the laborious and imprecise procedures for determining low levels of weak β-radiation emitters, ^{14}C or ^{3}H, in tissues where limited quantities of sample must be either dissolved in organic solvents for radioassay or oxidized for collection of $^{14}CO_2$ or $^{3}H_2O$. Most importantly, the high specific radioactivity allows study of low-activity tissues, and study of slowly catabolized proteins, or proteins at low concentration in plasma where injection of a large mass of protein is not appropriate.

We have shown in the present study that the radioiodinated TC label does meet the critical requirements for an intracellularly trapped label: recognition by cells is not altered, and the label from degraded protein is effectively trapped in cells. A caveat to the latter conclusion is that leakage of label from liver after uptake of some labelled proteins, e.g. asialofetuin, is high, but this leaked label can be accounted for in faeces and gut contents. Apparently re-uptake of this material by the gut is minimal.

The TC ligand is labelled to high specific radioactivity by using carrier-free radioiodide. Specific radioactivities at least 100 times those obtained with the [^{14}C]sucrose label are readily achieved. We have not tried to maximize specific radioactivity, but have on occasion used TC ligand labelled to the extent of almost 100 mCi of ^{125}I-μmol. At this level, efficiency of utilization of the radioiodide was more than 80% overall. If a protein of mol.wt. 50000 were to be labelled with this preparation to the extent of one TC ligand/protein molecule, it would represent a specific radioactivity of about 4000 d.p.m./ng of protein. The limitations in labelling small amounts of material to high specific radioactivity appear to lie in the chemical manipulation of small amounts of TC ligand and reagents, and in the possibility of radiation damage to the proteins.

We have used cyanuric chloride to link the TC ligand to protein. This compound contains three displaceable chlorine atoms; displacement of one de-activates the others; displacement of another further de-activates the last (Smolin & Rapaport, 1959; Chandhari & Bishop, 1972). We have previously used the same reagent to attach [^{14}C]-sucrose to protein. In that case, hydroxy groups of sucrose were the attacking nucleophile; in the present case we believe that the secondary amine of the TC ligand is the attacking agent. We were concerned that the reagent might cause protein cross-linking by virtue of the third displaceable chlorine atom, although direct attempts to demonstrate such activity of the third chlorine atom have been negative (Pittman *et al.*, 1979*a*). We found evidence of extensive cross-linking only when using [^{14}C]sucrose to label apo A1, a protein which self-associates strongly and may hold reacting moieties in configurations particularly favourable for the cross-linking reaction. We found moderate amounts of dimerization of apo A1 when using the TC ligand, and these had to be removed before study of the [^{125}I-TC]-apo A1 (Glass *et al.*, 1983). The lesser extent of cross-linking in the latter case may be explained by the greater de-activating effect of nitrogen compared with oxygen attached to the triazine ring of cyanuric chloride (Smolin & Rapaport, 1959; Chandhari & Bishop, 1972), or by the use of equimolar amounts of TC ligand and cyanuric chloride in the present case so that the chance of attaching two cyanuric chloride moieties to one TC ligand was small.

An attractive feature of the methodology reported here is the double-label approach used to correct the tissues' total content of TC label for that still on intact protein. It was found that direct radioassay of pieces of tissue was adequate for determining the activities of all tissues except those least active in a protein's catabolism. This does not mean that high absolute activity of a tissue is required to obtain satisfactory results by this simple method, but only that tissues of lowest specific activity in catabolism of any given protein will be underestimated because of residual radioactive iodide or iodotyrosine. If a protein is slowly catabolized overall, the body pool of radioiodide will be proportionately low. The low-activity tissues can be accurately corrected for their content of undegraded [^{125}I-TC],^{131}I-labelled protein by homogenization and determination of protein-bound ^{131}I, as in method 2 of Table 1.

It has been recognized that the trapped-label methodology has applications *in vitro* as well as *in vivo*. Since the catabolic products cannot cross membranes, they are trapped in the subcellular compartment in which they are produced. Thus the trapped label allows identification of the intracellular sites of degradation of proteins and the fates of the resulting labelled products. The [^{14}C]sucrose label has been used for such a purpose (Attie *et al.*, 1980; Tolleshaug & Berg, 1981).

There may also be clinical application of the trapped-label methodology. The radioiodinated TC ligand can be used with any targeted protein to deliver radioiodine to target cells and trap it there for therapeutic purposes or for radiological imaging. Several isotopes of iodine are available with various half-lives and decay modes to suit various purposes. As a therapeutic agent, trapping would allow concentration of large amounts of isotope in target cells and increase the exposure of those cells compared with other cells. As a radiological imaging agent the trapped label would intensify the image of target cells. The double-label approach might even be used to instrumentally subtract out the contribution of undegraded protein not yet incorporated into cells, and so further enhance the sensitivity of the imaging. It also is evident that moieties other than tyramine can be attached to cellobiose for trapping so that other radionuclides or therapeutic agents may be targeted to specific tissues and cell types based on their receptor systems or other cell-surface properties.

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