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Non-invasive detection of protein metabolism *in vivo* by n.m.r. spectroscopy

Application of a novel ^{19}F-containing residualizing label

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Protein residualizing labels facilitate localization of tissue sites of protein catabolism and the quantification of protein accumulation because of their prolonged intracellular retention times. Radioiodinated residualizing labels have been used to define the metabolism of a wide variety of proteins, but this has necessitated destructive analysis. Here we describe the implementation and validation of a novel ^{19}F-containing residualizing label for protein, *NN*-dilactitol-3,5-bis(trifluoromethyl)benzylamine (DLBA), that permits the non-invasive assessment of protein accumulation and catabolism by n.m.r. spectroscopy *in vivo*. DLBA comprises a reporter molecule containing six equivalent ^{19}F atoms. ^{19}F is strongly n.m.r.-active, has 100 % natural abundance, and is present in minimal background concentrations in soft tissues. We validated the use of DLBA as a protein-labelling compound by coupling to asialofetuin (ASF), a protein that is recognized exclusively by hepatic tissue via a saturable receptor-mediated process. Coupling of DLBA to ASF by reductive amination had no effect on the physiological receptor-mediated uptake of the protein in rat liver *in vivo*. The ^{19}F-n.m.r. spectrum of DLBA exhibited a single peak that was subject to a small chemical-shift change and broadening after coupling to ASF. Pronase digestion of DLBA–ASF was performed to simulate intracellular degradation products, and resulted in a narrower set of resonances, with chemical shifts intermediate between those of uncoupled DLBA and DLBA–ASF. Intravenous administration of DLBA–ASF to rats followed by quantification of ^{19}F in homogenates of liver tissue indicated that the half-life of residence time of degradation products from DLBA–ASF in liver was approx. 2 days. This intracellular half-life was comparable with that described for similar residualizing labels that contain radioiodide as a reporter. Similar results for the half-life of retention were obtained non-destructively and non-invasively *in situ* with the use of a whole-body radio-frequency antenna to acquire sequential spectra over 80 h after intravenous administration of DLBA–ASF. Quantification of these spectra demonstrated an initial accumulation of DLBA–ASF in liver followed by an expected gradual loss of ^{19}F-labelled degradation products. The approach developed offers promise for the sequential and longitudinal characterization of metabolism of specific proteins in individual experimental animals and ultimately in human subjects.

INTRODUCTION

Determination of the tissue sites and cellular mechanisms regulating the catabolism of specific proteins with prolonged half-lives in plasma has recently become feasible with the introduction of residualizing labels that are covalently coupled to proteins of interest (Strobel *et al.*, 1985; Pittman & Taylor, 1986; Maxwell *et al.*, 1987, 1988; Baynes *et al.*, 1988). The glycoconjugate labels used are transported into cells by the carrier protein without distortion of the physiological mechanisms responsible for the uptake process. After catabolism of the protein, glycoconjugate-labelled degradation products are entrapped within lysosomes. Thus, although the carrier protein may be removed from the circulation slowly, the gradual intracellular accumulation of labelled catabolites permits identification of tissue sites of removal. The persistence of glycoconjugate-labelled degradation products in intracellular compartments results from their size, polarity and resistance to degradation by lysosomal hydrolases (Strobel *et al.*, 1985; Pittman & Taylor, 1986; Maxwell *et al.*, 1987, 1988; Baynes *et al.*, 1988).

We have described previously the synthesis of residualizing labels in which selected reporter molecules are attached to lactose and the resultant glycoconjugate is coupled to protein by reductive amination (Strobel *et al.*, 1985; Maxwell *et al.*, 1987). The most commonly used residualizing protein label that is coupled by reductive amination is radioiodinated dilactitol-tyramine. Radioiodinated residualizing labels have been used to characterize the metabolism *in vivo* and *in vitro* of a variety of

Abbreviations used: ASF, asialofetuin; BA, 3,5-bis(trifluoromethyl)benzylamine; DLBA, *NN*-dilactitol-3,5-bis(trifluoromethyl)benzylamine.

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proteins, including lipoproteins (Pittman *et al.*, 1983; Daugherty *et al.*, 1985), asialofetuin (ASF) (Strobel *et al.*, 1985; Maxwell *et al.*, 1988), albumin (Yedgar *et al.*, 1983; Strobel *et al.*, 1986) and immunoglobulins (Moldoveanu *et al.*, 1988; Weis *et al.*, 1988). A fluorescent residualizing label has been used to define characteristics of albumin metabolism (Baynes *et al.*, 1988). Neither the chemical means used to attach the residualizing labels to proteins via reductive amination nor the nature of the labels has been found to affect either the physical or biological properties of any of the proteins and enzymes studied. In the present study, a new label is described that contains the same residualizing entity as that in the glycoconjugates described previously, but incorporates the highly n.m.r.-active ^{19}F nuclide as a reporter.

One of several advantages of non-invasive determination of protein metabolism by n.m.r. spectroscopy over more conventional procedures is the non-radioactive nature of n.m.r.-active nuclides. Thus experimental procedures are not complicated by restrictively short isotope half-lives. There are no attendant health risks to be expected associated with the use of n.m.r.-active nuclides for human subjects. Use of the spin-$\frac{1}{2}$ ^{19}F nuclide for n.m.r. spectroscopy has additional advantages, including its high sensitivity of detection (0.83 relative to ^{1}H), 100% natural abundance and an atomic radius comparable with that of hydrogen, which facilitates incorporation into many compounds. In addition, label-to-background signal ratios are favourable for fluorinated compounds *in vivo* because of minimal concentrations of endogenous ^{19}F-containing compounds in soft tissues. The feasibility for detection of ^{19}F quantitatively, sequentially and non-invasively offers promise for the characterization of the rate and extent of protein catabolism in selected tissues of an individual animal.

The present paper describes the synthesis and use of a ^{19}F-containing residualizing label, *NN*-dilactitol-3,5-bis-(trifluoromethyl)benzylamine (DLBA), that is amenable to use in non-invasive detection of protein metabolism by n.m.r. spectroscopy *in vivo*. The usefulness of this approach for metabolic studies is demonstrated by the detection of the accumulation and catabolism of DLBA-labelled ASF *in vivo*, a protein that is removed from plasma exclusively by the liver (Ashwell & Harford, 1982).

MATERIALS AND METHODS

Materials

Lactose, galactose oxidase, Pronase, 99% $^{2}H_2O$, neuraminidase and $NaBH_3CN$ were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.), and 3,5-bis-(trifluoromethyl)benzylamine (BA), hexafluorobenzene and trifluoroacetic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). ASF was prepared from fetuin (Gibco Laboratories, New York, NY, U.S.A.) by treatment with insolubilized neuraminidase followed by gel-exclusion chromatography on either Sephacryl S200 or Superose 12 (Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.).

Synthesis and isolation of DLBA

Reaction mixtures containing BA (50 mM), lactose (250 mM) and $NaBH_3CN$ (200 mM) in methanol (5 ml) were incubated at 65 °C for 2 h with occasional mixing, then cooled and centrifuged to pellet undissolved lactose. The supernatant fraction was acidified with 1 M-HCl (5 ml) to discharge residual $NaBH_3CN$, concentrated by rotary evaporation and redissolved in deionized water (3–4 ml). Reversed-phase h.p.l.c. was performed with a Varian model 5000 liquid chromatograph, as described previously (Baynes *et al.*, 1988), with the use of the following buffers: solvent A, aq. 0.1% (v/v) trifluoroacetic acid; B, 50% acetonitrile (v/v) in aq. 0.1% (v/v) trifluoroacetic acid. The following gradient was used to elute materials from the column: 0 min, 10% (v/v) solvent B; 5 min, 25% (v/v) solvent B; 25 min, 50% (v/v) solvent B; 30 min, 100% (v/v) solvent B.

Coupling of DLBA to ASF

DLBA (3 μmol) in 0.2 M-potassium phosphate buffer, pH 7.7 (0.3 ml), was oxidized at 37 °C by incubation with galactose oxidase in 0.1 M-phosphate buffer, pH 7.0 (5 μl); 10 units of galactose oxidase were added hourly until a total of 40 units were present in the incubation mixture. The oxidation reaction was judged to be approx. 30% efficient by the bicinchoninic acid assay for reducing sugars (McFeeters, 1980) with galactose as a standard. Efficiency was not improved by the addition of additional enzyme or by longer intervals of incubation. ASF was added to the incubation mixture at 10% the level of aldehyde, i.e. a ratio of DLBA-aldehyde to ASF of 10:1 (mol/mol). $NaBH_3CN$ was added to give a final concentration of 36 mM and the coupling reaction was incubated at 37 °C for 5 h. Labelled protein was separated from unbound label by passage through a column (1.2 cm × 30.0 cm) containing either Sephacryl S200 or Superose 12. Proteolytic digestions of DLBA–ASF were performed by incubating protein (4 mg) with Pronase (1%, w/v) in 0.5 M-potassium phosphate buffer, pH 7.7, for 48 h at room temperature.

Characterization of DLBA–ASF metabolism

Rats (130–170 g body wt.) were given intracardiac injections of ASF as described previously (Strobel *et al.*, 1985). To determine the actual amount of protein injected, fluorescein isothiocyanate-labelled rat serum albumin (Arnold *et al.*, 1975) was co-injected with DLBA–ASF and blood samples were obtained from the tail vein during the first 10 min after injection. Typically, 80% of expected fluorescence was recovered in plasma, based on the assumption that plasma volume was equal to 3.5% of body weight (Maxwell *et al.*, 1987). At selected intervals after injection, animals were exsanguinated by perfusion with phosphate-buffered saline (Dulbecco & Vogt, 1974) and the liver of each was removed and homogenized. The concentrations of label in liver homogenates were determined by ^{19}F-n.m.r. spectroscopy.

Analysis of DLBA–ASF and degradation products by high-resolution ^{19}F n.m.r.

^{19}F-n.m.r. spectroscopy of DLBA, DLBA–ASF and Pronase digests of DLBA–ASF was performed with a Varian VXR-500 spectrometer operating at 470.3 MHz. Measurement of spin–lattice relaxation time (T_1) was performed with the inversion-recovery method, resulting in T_1 determinations of 1.0 s and 0.8 s for DLBA and DLBA–ASF respectively. Chemical shifts were externally referenced to trifluoroacetic acid and are reported according to I.U.P.A.C. convention (I.U.P.A.C. Recommendations on NMR Spectra, 1976). The degree of substitution of protein with DLBA was determined by

comparison of integrated areas of the fluorine resonances of DLBA–ASF and external TFA of known concentration under fully relaxed conditions. ^{19}F-n.m.r. spectra of DLBA–ASF in rat liver homogenates prepared at selected intervals after injection were obtained by use of a Bruker AM-300 spectrometer operating at 282.3 MHz. Relative concentrations of ^{19}F-containing residualizing label retained in livers were determined by comparison of integrated resonance areas.

^{19}F-n.m.r. spectroscopy *in vivo*

Male Sprague–Dawley rats (120–140 g body wt.) were deprived of food for 12–18 h and anaesthetized with a mixture of ketamine (20 mg/kg intramuscularly, and xylazine (50 mg/kg intramuscularly). DLBA–ASF was administered intravenously via a femoral vein. The liver was localized initially by surgical exposure in combination with surface-coil spectroscopy as described previously (Ackerman *et al.*, 1987). Briefly, an approx. 4 cm transverse subcostal incision was made in the abdomen to expose the liver and the falciform ligament was cut. The animal was wrapped in plastic film to prevent moisture loss and secured in the n.m.r. probe in a vertical position. The surface coil was placed against a 1 mm-thick glass plate positioned over the liver. Body temperature was maintained constant with the use of a circulating water jacket. Surface-coil ^{19}F-n.m.r. experiments were performed without proton decoupling at 188.2 MHz with a Bruker CXP-200 spectrometer. A triple-turn 2 cm-diameter surface-coil antenna characterized by a 90° pulse width at the coil centre of 15 μs at 100 W was used. Signal-to-noise ratio was optimized by use of a pulse width of 6 μs and a pulse repetition period of 0.1 s. A spectral width of 50 kHz and acquisition time of 0.04 s were used. Sequential 30 min spectra were acquired from 30 min to 4–8 h after injection. The static-field homogeneity was optimized by shimming on the free induction decay of tissue water protons with the fluorine antenna.

Non-invasive ^{19}F-n.m.r. quantification of metabolism of DLBA–ASF was performed with an SIS 200/400 horizontal magnet system operating at 188.2 MHz. A homogenous antenna consisting of a one-turn-in-parallel saddle-shaped coil was placed around the body of each rat over the area that included the entire liver. The coil contained a fixed ^{19}F-external-reference capillary of hexafluorobenzene (diluted in benzene with chromium acetylacetonate as a relaxing agent) that permitted quantification of accumulation and retention of the ^{19}F-containing residualizing label in the liver by normalization of integrated ^{19}F resonance areas to the external standard. The 90° pulse width was determined before each measurement and ranged between 36 and 38 μs at 80 W. Spectra were acquired for 30–60 min under rapid pulse-repetition conditions with a sweep width of 30 kHz, acquisition time of 0.068 s, optimized excitation flip angle of 28° and pulse repetition period of 0.1 s. All spectra derived from studies *in vivo* were processed with a 100–200 Hz exponential filter.

All procedures involving the use of animals were approved by the Washington University Committee for the Humane Care of Animals.

RESULTS AND DISCUSSION

The recent implementation of protein residualizing labels has permitted the quantification and characterization of the catabolic processes of macromolecules in tissues *in vivo* that could not be achieved with conventional radiolabels. Although the presently available residualizing protein labels offer considerable advantages, their use requires dissection of the tissue of interest, and consequently serial autopsy is required to acquire sequential data. To overcome this shortcoming, we undertook the study of a novel ^{19}F-containing residualizing label, DLBA, that may be detected by n.m.r. spectroscopy, thus facilitating the acquisition of sequential data from an individual animal without the need for invasive procedures.

The chromatogram in Fig. 1(*a*) illustrates the major products recovered in the reaction mixture used to prepare DLBA. Only a small percentage of residual BA was detected. The chemical composition of the materials eluted at approx. 14 and 23 min indicated that these were the di- and mono-lactitol derivatives of BA. The order of elution was similar to that for other mono- and di-substituted glycoconjugates purified by reversed-phase h.p.l.c. (Baynes *et al.*, 1988). The structure of the purified compound shown in Fig. 1(*b*) was confirmed by mass spectrometry (Fig. 2); the structure of the monolactitol derivative was confirmed by mass spectrometry (results not shown).

The n.m.r. spectrum of purified DLBA shown in Fig. 3(*a*) showed a single peak at 12.9 p.p.m. Compared with DLBA, the DLBA–ASF (DLBA/ASF molar ratio approx. 3:1) demonstrated a 0.3–0.7 p.p.m. shift toward a higher frequency (less shielding). The increased linewidth and structure of the spectrum are suggestive of the heterogeneity in chemical shift of at least three non-equivalent DLBA-binding sites of the protein (Fig. 3*b*). Indeed, after Pronase digestion of DLBA–ASF the spectrum shows a narrower set of resonances, with

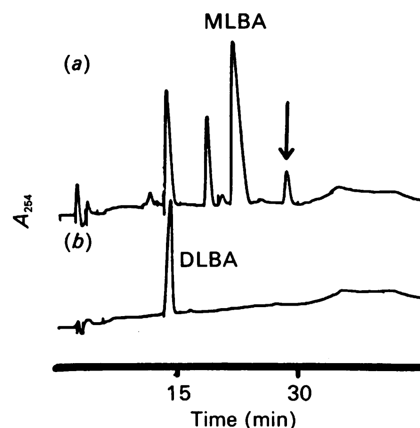

Fig. 1. Analysis of reaction mixture used to prepare DLBA

(*a*) A portion of the reaction mixture (5 μl, 1.5 μmol of starting BA) prepared as described in the Materials and methods section was analysed by h.p.l.c. The arrow indicates the elution position of BA. On the basis of the anthrone assay for carbohydrate (Spiro, 1966) and absorbance at 254 nm (ϵ_{254} 2.3 × 10^5 $M^{-1} \cdot cm^{-1}$), material eluted at approx. 15 min (DLBA) and 23 min {MLBA, [*N*-lacitol-3,5-bis(trifluoromethyl)benzylamine]} contained carbohydrate and BA in molar ratios of 2.1:1 and 1:1 respectively. (*b*) Material eluted at 15 min was pooled for several runs and re-injected onto the h.p.l.c. for analysis of purity.

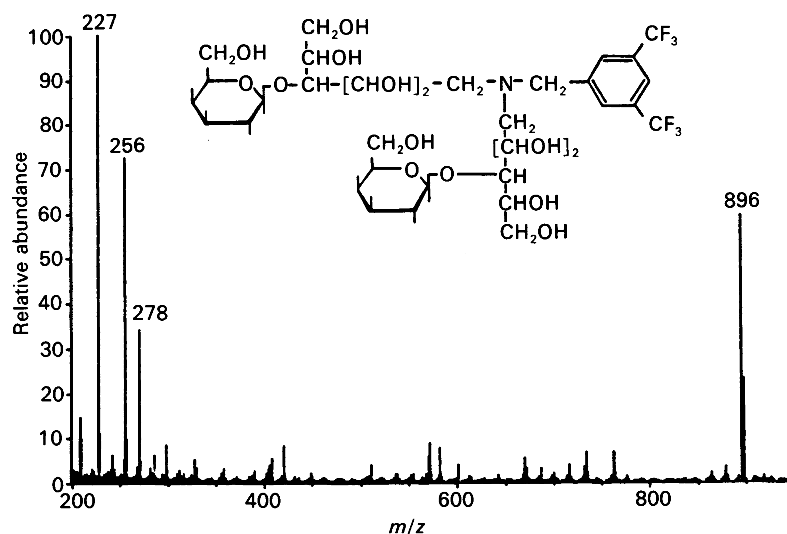

Fig. 2. Mass spectrum of purified DLBA

A portion of the sample shown in Fig. 1(*b*) was dissolved in diglycerin and analysed by fast-atom-bombardment m.s. in a VG model 70SQ mass spectrometer (VG Instruments, Manchester, U.K.). Assignments: m/z 896 = M+H; m/z 256 = [CH_2=NH(CH_2)–$C_6H_3(CF_3)_2$]; m/z 227 = [CH_2=$C_6H_3(CF_3)_2$].

linewidth and chemical shift intermediate between those of free DLBA and coupled DLBA–ASF (Fig. 3*c*).

In previous studies with residualizing labels, proteins have been conjugated with at least 1 mol of label/mol of protein. This low substitution was performed to limit possible alterations in protein structure and to ensure that there was no hindrance of protein–cell interactions. To increase the sensitivity of detection by n.m.r. spectroscopy, ASF was substituted with up to 3 mol of DLBA/mol of protein. It was therefore necessary to establish that the normal, receptor-mediated, uptake of the protein (Ashwell & Harford, 1982) was not affected by this higher degree of substitution. DLBA–ASF (molar ratio 3:1) was radiolabelled with ^{125}I and 20 μg was injected via a femoral vein into anaesthetized rats. The half-life in plasma of the protein was in the region of 50–100 s, which is consistent with previously published reports. When this DLBA–^{125}I-ASF was co-injected with a competing dose of 3.5 mg of native ASF, the half-life in plasma was extended to more than 30 min (results not shown). The results of these competition experiments indicated that uptake of DLBA–ASF was mediated by the asialoglycoprotein receptor in hepatocytes (Ashwell & Harford, 1982); the rate of uptake was not significantly affected by the attachment of 3 mol of DLBA/mol of ASF.

Each molecule of DLBA contains six equivalent fluorine atoms. Thus the ability to achieve multiple substitution without alteration of mechanisms responsible for cellular interactions provided a convenient means for delivering substantial quantities of fluorine to liver (up to 18 mol/mol of ASF) without injection of a large mass of protein.

As a first step in evaluating whether DLBA could serve as a residualizing label for n.m.r. spectroscopy, the retention of ^{19}F was quantified in homogenates of liver tissue prepared at selected intervals after intravenous administration of DLBA–ASF to rats. The spectrum in Fig. 4 was obtained from a homogenate of a liver that had been excised 3 h after administration of DLBA–ASF. Its profile was similar to that of spectra obtained with homogenates of livers prepared 24 and 48 h after administration of DLBA–ASF (spectra not shown). Integrated areas of spectra taken at the selected intervals were

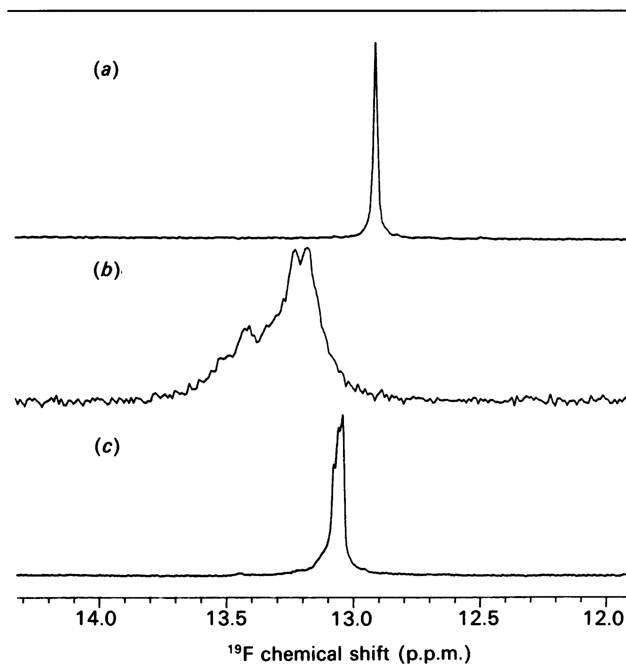

Fig. 3. ^{19}F-n.m.r. spectra of DLBA and DLBA conjugated to ASF

Spectra were acquired as described in the Materials and methods section. Chemical shifts are presented in p.p.m. downfield from trifluoroacetic acid. (*a*) ^{19}F-n.m.r. spectrum of a solution containing DLBA (399 nmol/ml). (*b*) ^{19}F-n.m.r. spectrum of a solution containing DLBA conjugated to ASF (11 mg/ml). As a control, native ASF was analysed under identical conditions. As expected, no ^{19}F signals were detected. (*c*) ^{19}F-n.m.r. spectrum of Pronase-digested DLBA–ASF (5 mg/ml).

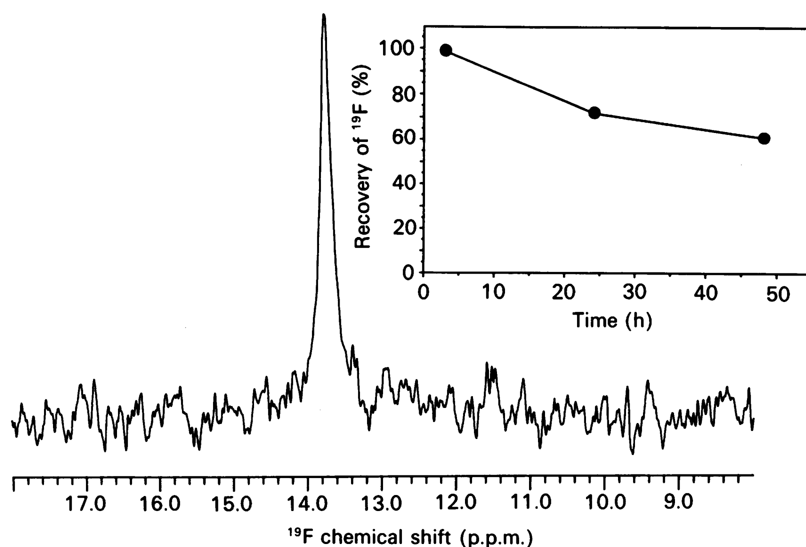

Fig. 4. ^{19}F-n.m.r. spectrum *in vivo* of DLBA–ASF in liver determined by invasive procedures

At 3 h after intravenous administration of DLBA–ASF (2.5 mg; 3.3 mol of DLBA/mol of ASF) to a rat, the liver was removed and a 1 g segment was homogenized with 99 % $^{2}H_{2}O$ (0.5 ml). Spectra of the homogenates were acquired for 13 h. No ^{19}F signal was detected in a liver homogenate from a control animal after 6 h of acquisition under the same conditions. The inset shows the recovery of n.m.r.-active ^{19}F in homogenized hepatic tissue as a function of time after administration of DLBA–ASF. The n.m.r.-active ^{19}F recovered at the first interval was taken as 100 %. Each point represents the mean of two values from animals at each interval.

adjusted for the dose administered and for the weight of liver. Consideration of these variables permitted estimation of the recovery of n.m.r.-active ^{19}F in hepatic tissue at the selected intervals after injection. Degradation products from DLBA–protein were effectively retained in liver with a half-life in tissue of about 2 days (Fig. 4). This half-life of retention within tissues is comparable with that for other residualizing labels of similar structure (but different reporter molecules) that are coupled to protein via reductive amination (Strobel *et al.*, 1985; Maxwell *et al.*, 1987).

Delineation of accumulation of DLBA–ASF in rat liver *in vivo* was initially accomplished by surface-coil ^{19}F-n.m.r. spectroscopy with surgical exposure of the liver to maximize sensitivity of detection. The accumulation of ^{19}F in liver was observed by n.m.r. after intravenous administration of DLBA–ASF (7 mg). A ^{19}F-n.m.r. spectrum acquired at 4–4.5 h after injection showed a resonance at 14 p.p.m. with a good signal-to-noise ratio. Thus the ^{19}F-n.m.r. spectrum of DLBA–ASF was readily detected with data accumulation times as short as 30 min (Fig. 5). As expected, the linewidth *in vivo*, of 188.2 MHz, was broader (approx. 3 times) the linewidth *in vitro* of DLBA–ASF. The localization of the ^{19}F-n.m.r. signal to hepatic tissue was confirmed by the absence of any discernible resonance 1 h after 1 h of data collection when the surface coil was positioned on the animal more than one coil radius from the liver.

The time course of DLBA–ASF metabolism was determined in hepatic tissue quantitatively and non-invasively with a coil that detected ^{19}F signal from the entire liver in the anaesthetized rat. Spectra were acquired at selected intervals up to 80 h after intravenous injection of DLBA–ASF (5–7.5 mg). For these experiments the injected DLBA–ASF conjugate was substituted with between 1 and 3.6 mol of label/mol of protein. The sensitivity of this configuration was more than adequate for quantification of the residualizing label, as exemplified by the spectrum, shown in Fig. 6, that was acquired 8.5 h after injection. Integrated areas of ^{19}F resonances, normalized to the external standard of hexafluorobenzene, were plotted as a function of time to yield the time course of accumulation and retention of DLBA–ASF in hepatic tissue (Fig. 6). Among the intervals selected, the highest concentration of the label in liver was observed 9 h after injection. After this maximum, signal decreased with time, yielding an estimated half-life of ^{19}F within the hepatic tissue of approx. 2 days. As shown by the inset in Fig. 6, the kinetics of the accumulation and release of DLBA–ASF and its degrad-

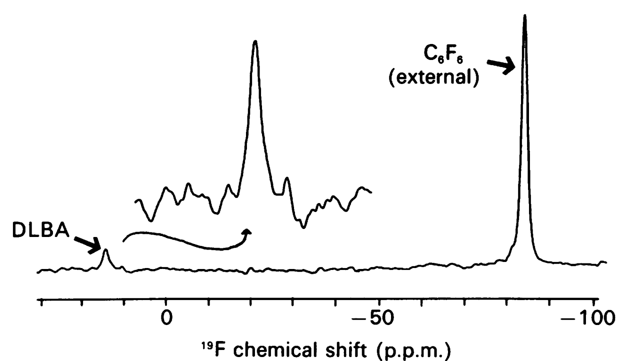

Fig. 5. ^{19}F-n.m.r. spectrum obtained from a surgically exposed liver by surface-coil antenna 4 h after injection of DLBA–ASF (7 mg, substitution approx. 1 mol of DLBA/mol of ASF)

The ^{19}F-n.m.r. spectrum was acquired in 30 min from a rat liver *in situ* 4–4.5 h after intravenous administration of DLBA–ASF. The resonance from DLBA has been expanded by a factor of 8-fold in the inset.

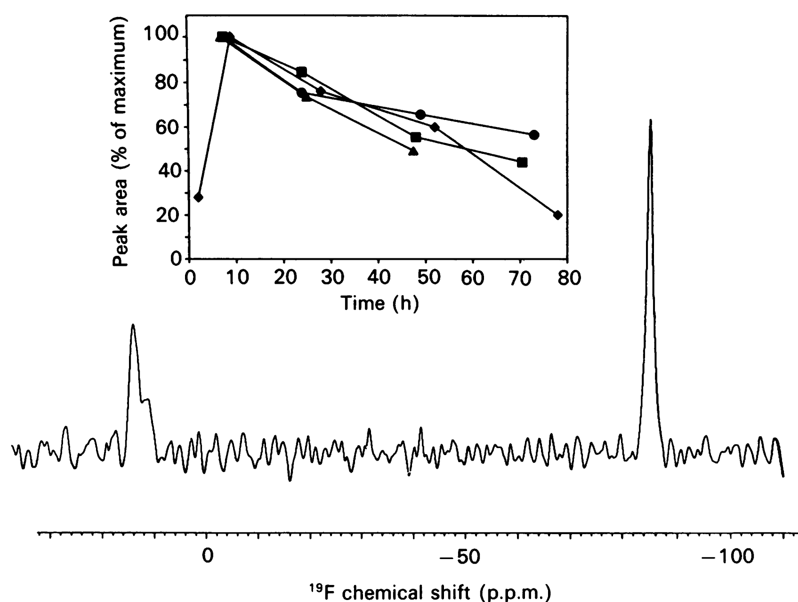

Fig. 6. ^{19}F-n.m.r. spectrum obtained non-invasively from livers of anaesthetized rats by a homogeneous coil after injection of DLBA–ASF (5–7.5 mg)

Spectra were obtained at selected intervals up to 80 h. The acquisition time for each spectrum was between 15 and 60 min. The residualizing label is present to the extreme left and the external reference signal of hexafluorobenzene is to the extreme right. The baseline was corrected to a third-order polynomial fit. The inset shows the quantification of ^{19}F present in the liver of the four animals examined as a function of time expressed as a percentage of the maximum peak area. Maximum peak area was recorded at approx. 8 h in all cases.

ation products were highly reproducible in the four animals examined. This half-life in tissues of DLBA-labelled degradation products is consistent with the retention time determined by destructive analysis and with half-lives of other residualizing labels of similar structure (Maxwell *et al.*, 1987).

Our studies were undertaken to define the feasibility of the use of n.m.r. spectroscopy non-invasively for detection of protein metabolism *in vivo*. The experiments employed a model system that would optimize delivery of ^{19}F to a single organ. These data indicate that delivery of about 1 μmol of ^{19}F to rat liver is more than adequate for sensitive detection of protein catabolites and validate the use of a novel ^{19}F-containing residualizing label for the non-invasive characterization of protein metabolism *in vivo*.

Although the sensitivity of the approach developed was adequate for the purposes of the present study, its future application will be facilitated by optimization of coil design, improvements in signal-processing techniques (Bretthorst *et al.*, 1988*a*,*b*) and the attachment of other reporter groups to the residualizing disaccharide to increase the number of equivalent fluorine atoms. The method is applicable to the sequential and longitudinal characterization of the rates and extent of catabolism of specific proteins in specific tissues in individual experimental animals and may ultimately be implemented in human subjects.

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