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[^{3}H]Raffinose, a Novel Radioactive Label for Determining Organ Sites of Catabolism of Proteins in the Circulation*

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The primary tissue sites of catabolism of plasma proteins with long circulating half-lives are unknown. It has been difficult to identify these sites because plasma proteins are delivered to tissues at relatively slow rates but are rapidly degraded intracellularly within lysosomes. Therefore, a tracer attached to protein is lost from the site of uptake before an amount sufficient for quantitation can accumulate. We hypothesized that sucrose (Glu$p\alpha$1-2 βFru$f$) would be a useful label to circumvent this difficulty because of the stability of sucrose in lysosomes; and thus, sucrose should remain in tissue long after the protein to which it was attached had been degraded to products released from the lysosome. [G-^{3}H]Raffinose (RAF, Gal$p\alpha$1-6 Glu$p\alpha$1-2βFru$f$) was selected as the vehicle for attaching sucrose to protein. [^{3}H]RAF was converted to the C-6 aldehydogalactose form with galactose oxidase and then covalently coupled to protein by reductive amination using $NaBH_3CN$.

[^{3}H]RAF was coupled first to two relatively long lived plasma proteins, bovine serum albumin and fetuin. The half-lives of these proteins in the rat circulation ($t_{1/2}$ = ~24 h) was unchanged, suggesting that RAF did not alter the normal mechanisms of protein clearance. When attached to short lived proteins with known sites of catabolism, such as asialofetuin, RNase B, and heat-denatured albumin, neither the tissue nor cellular sites of uptake of the proteins were altered. Thus, [^{3}H]RAF-asialofetuin was recovered almost exclusively (>90%) in the liver parenchymal cell fraction, while both [^{3}H]RAF-labeled RNase B and heat-denatured albumin were recovered primarily (>85%) in nonparenchymal cells. In addition, the RAF label was observed to reside stably ($t_{1/2}$ > 100 h) in the liver following degradation of the carrier protein; in contrast, radioactivity from ^{125}I-labeled asialofetuin or RNase B was rapidly ($t_{1/2}$ < 30 min) lost from liver. Radioactivity from [^{3}H]RAF-proteins was recovered in a lysosomally enriched subcellular fraction in liver and consisted of a low molecular weight species (~1100), containing both glucose and fructose in a ratio similar to that in the original protein. The results of these studies establish that [^{3}H]RAF covalently coupled to plasma proteins should be a useful radioactive tracer for detecting the tissue and cellular sites of catabolism of long lived circulating proteins.

There is extensive information concerning the sites of synthesis and kinetics of turnover of plasma proteins; however, there is little firm evidence concerning their mechanisms of clearance from the circulation or their tissue sites of degradation (2–4). Perhaps the greatest technical difficulty in obtaining this information has been the absence of a suitable radioactive label with which to monitor the sites of catabolism of proteins with the long circulating half-lives characteristic of plasma proteins. In general, the rate of intracellular degradation of plasma proteins within lysosomes far exceeds the rate of clearance of these long lived proteins. Thus, if plasma proteins are labeled with conventional labels, such as ^{125}I or radioactive amino acids, the tracer does not accumulate at the site of catabolism, but, rather, as the protein is degraded to monomeric products which exit from the lysosome, the label is lost to cellular or extracellular metabolic pools, or both. In contrast, the sites of catabolism of proteins with short circulating half-lives are readily identified since their rates of clearance are relatively rapid, compared to the rate of lysosomal digestion. It has been possible, therefore, to identify the liver as the primary site for catabolism of [^{3}H,^{64}Cu]asialoceruloplasmin (5) and ^{125}I-RNase B (6) injected in rats, and bovine β-glucuronidase injected in mice (7), all proteins which exhibit half-lives of 15 min or less.

In order to circumvent the difficulties inherent in determining the *in vivo* fate of long lived plasma proteins, a tracer is needed which will 1) not affect mechanisms of protein clearance and catabolism, 2) be resistant to degradation by lysosomal hydrolases, and 3) accumulate in lysosomes at the site of the labeled protein's degradation. We hypothesized that sucrose (Glc$p\alpha$1-2βFru$_f$) covalently attached to protein through its glucosyl moiety might fulfill these criteria for the following reasons. First, because of the stable equilibration of inulin (a fructan) and raffinose (RAF, Gal$p\alpha$1-6Glc$p\alpha$1-2βFru$_f$) into plasma and extravascular spaces *in vivo* (8), it seemed unlikely that the terminal fructose residues on a protein would serve as recognition markers for a carbohydrate-dependent clearance process analogous to those already described for galactose (9), fucose (10), mannose (11, 12), and *N*-acetylglucosamine (13, 14) terminal glycoproteins. Second, because of the absence of significant levels of mammalian lysosomal sucrase activity (15), it seemed unlikely that hydrolysis of the sucrose moiety would occur either before or after degradation of the protein. Finally, since sucrose does not readily diffuse from lysosomes (15), it or its amino acid conjugate should accumulate at this subcellular site of protein

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catabolism, despite the fact that other components of the protein would exit from the lysosome following degradation.

As a vehicle for the attachment of sucrose to protein, we chose the trisaccharide [G-^{3}H]raffinose as our radioactive starting material. Oxidation of the C-6 hydroxyl of the galactose residue, using galactose oxidase (16), provided an aldehyde function which could be readily coupled to protein, under mild conditions, using $NaBH_3CN$ (17). We present below the results obtained *in vitro* for the coupling of RAF[1] to several proteins by this method. We also present our results from studies on the comparative fates of ^{125}I- and [^{3}H]RAF-labeled proteins *in vivo*, as a test of our hypothesis that RAF attached to protein would be a stable, inert, label useful for determining the organ and cellular sites of plasma protein catabolism.

EXPERIMENTAL PROCEDURES

Materials—The following materials were purchased from the indicated sources: [G-^{3}H]RAF (~1500 mci/mmol) and Na^{125}I (New England Nuclear); ^{125}I-human serum albumin, (Mallinckrodt Laboratories); RAF, Glu, Gal, and Fru (P & L Laboratories); galactose oxidase, catalase, insolubilized neuraminidase, and $NaBH_3CN$ (Sigma). Bovine serum albumin (Pentex Laboratories) was heat-denatured according to the method of Cohen and Gordon (18). RNase B (Sigma type XII-B) was assayed, purified, and iodinated as previously described (6, 11). Asialofetuin was prepared from fetuin (Grand Island Biologic Co.) using insolubilized neuraminidase at 25°C for 18 h in 0.1 N sodium acetate, pH 5.2; sialic acid release was 75% of theoretical as judged by the thiobarbituric acid assay (19). ^{125}I-labeled albumin, fetuin, and asialofetuin were prepared using the lactoperoxidase technique (20) and reisolated by chromatography on Bio-Gel P-150 (Bio-Rad Co.).

Coupling of RAF to Protein—A typical reaction mixture for the conversion of RAF to raffinaldehyde contained 1 μmol of RAF, 20 units of galactose oxidase (150 μg of protein), and 130 units of catalase (10 μg of protein) in 1 ml of 0.1 N potassium phosphate buffer, pH 7.5, and was incubated for 4 h at 37°C. Using the Nelson-Somogyi procedure (21) to monitor aldehyde production, 75 to 80% of the theoretical amount of aldehyde was generated under these conditions (16). Longer times of incubation, further additions of enzyme, or increasing the initial amount of enzyme did not alter the yield of aldehyde. Aldehyde generated in this manner was stable to freezing for at least 10 days. In the coupling procedure, protein in 0.1 N potassium phosphate, pH 7.5, was mixed with 1 to 3 molar equivalents of aldehyde, and then $NaBH_3CN$ was added in the same buffer to a final concentration of 1 mg/ml (17 mM); coupling was carried out for 18 to 24 h at room temperature in a sealed tube. The kinetics or extent of coupling were determined in aliquots removed at desired times by measuring radioactivity or anthrone-positive (21) material, precipitable by acid, or both. Albumin was precipitated by the addition of an equal volume of 10% trichloroacetic acid; fetuin and RNase B were precipitated with 5% phosphotungstic acid in 2 N HCl, and 2.5% uranyl acetate in 12.5% trichloroacetic acid, respectively. Radiolabeled protein was isolated from incubation mixtures by chromatography on Bio-Gel P-150 (albumin, fetuin) or Sephadex G-75 (Pharmacia, RNase B) columns in 0.1 N ammonium acetate. Column effluents were monitored for absorbance at 280 nm or radioactivity, or both, and appropriate fractions were pooled, lyophilized, and redissolved in 0.9% NaCl for injection experiments.

In Vivo Experiments—Experiments were conducted in male Sprague-Dawley rats (175 to 225 g) under ether anesthesia. Experiments with RNase B were carried out in nephrectomized animals as previously described (6). For half-life determinations, proteins were injected intracardially and blood samples were removed at timed intervals from lateral tail veins. After centrifugation, radioactivity was determined in measured plasma aliquots. Organs were thoroughly perfused with saline (0.9% NaCl solution) before sampling for radioactivity determinations (6). For measurement of ^{3}H radioactivity, weighed aliquots of tissue were homogenized in 4 volumes of H_2O, and 0.1- to 0.2-ml aliquots were solubilized overnight at 25°C in 0.25 ml of 20% sodium dodecyl sulfate; when decolorization was needed, 10 to 20 μl of 30% H_2O_2 were added and samples were heated at 50°C. ^{3}H radioactivity was counted in a Triton X-100 toluene-based scintillation fluid (Beckman Bio-Solv EP). Weighed portions (~1 g) of tissue from ^{125}I-protein injection experiments were counted directly in a Beckman Gamma 4000 gamma counter. Isolation of parenchymal and non-parenchymal cells of liver by collagenase digestion and differential centrifugation was carried out as previously described (6). Subcellular fractionation of liver by differential centrifugation into nuclear, mitochondrial/lysosomal, and supernatant fractions was performed according to Gregoriadis and Sourkes (22) using β-*N*-acetylhexosaminidase activity (23) as a lysosomal marker enzyme.

In order to characterize products resulting from the *in vivo* degradation of [^{3}H]RAF-labeled proteins, livers were taken at various times postinjection of heat-denatured albumin or asialofetuin; the livers were homogenized in 4 volumes of H_2O, frozen, and thawed four to five times and then centrifuged at 12,000 × *g* for 30 min. The supernatant, containing 90 to 100% of the original radioactivity, was then passed through an Amicon PM-10 filter (nominal molecular weight cut off, 10,000), and 95 to 100% of the supernatant radioactivity was recovered. The ultrafiltrate was then lyophilized, redissolved in a small volume of 0.1 M ammonium acetate, and chromatographed on a Bio-Gel P2 column. Radioactive peaks were pooled and lyophilized, and their content of radioactive sugars was determined following hydrolysis in 2.0 N trifluoroacetic acid at 110°C for 3 h. Component sugars were separated by paper chromatography in a butanol/pyridine/water (9:5:4) solvent system and visualized using a sodium periodate/silver nitrate staining procedure (24). Radioactivity was located and quantitated by counting 5-mm sections of the paper chromatogram directly in scintillation fluid containing 10% H_2O.

RESULTS

In Vitro Experiments: Protein Modification

The overall scheme for the production of raffinaldehyde and its coupling to protein is shown in Fig. 1. As indicated, attachment of carbohydrate to protein by reductive amination required the presence of an aldehyde function on the sugar. This aldehyde function was introduced at the C-6 position of the galactose moiety of RAF using galactose oxidase. Catalase was included in the incubation mixture in order to destroy H_2O_2 produced during the oxidation reaction (16). Because of the selectivity of $NaBH_3CN$ for reduction of Schiff bases above pH 5 (25), the coupling reaction theoretically could be carried out simultaneously with the generation of aldehyde. However, since preliminary experiments indicated that $NaBH_3CN$ was inhibitory to galactose oxidase, the two reactions were carried out sequentially. A pH of 7.5 was chosen as the pH for coupling in order to keep plasma proteins close to physiologic pH and yet have conditions alkaline enough to

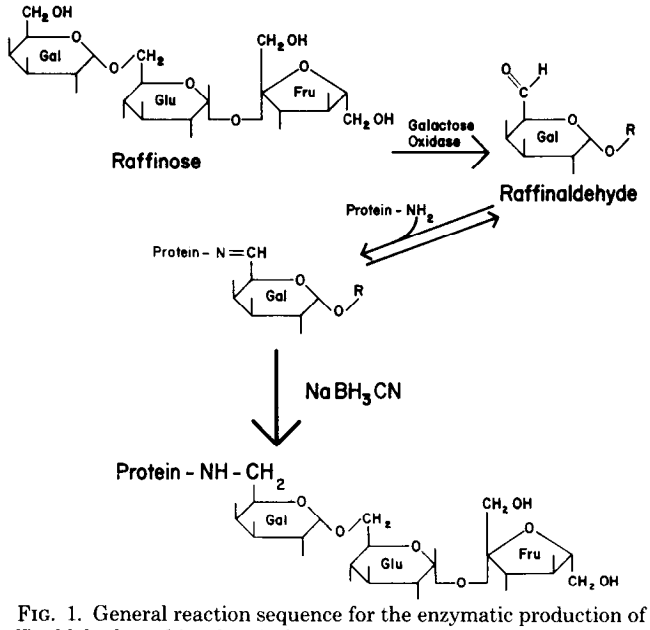

FIG. 1. General reaction sequence for the enzymatic production of raffinaldehyde and its chemical attachment to protein.

[1] The abbreviation used is: RAF, raffinose.

allow coupling to proceed at a reasonable rate (17, 26). The gel chromatographic profiles of modified proteins were identical to those of the starting preparations; RNase B enzymatic activity was unaffected by attachment of approximately 0.1 mol of RAF/mol of protein.

The kinetics of attachment of RAF to albumin under the conditions chosen as standard are illustrated in Fig. 2. The reaction was found to proceed at a relatively rapid rate for approximately 4 h and then continued more slowly thereafter. It should be noted that the amount of protein-bound carbohydrate (acid-precipitable) was consistently found to be somewhat greater when determined by the anthrone method (21) than by radioactivity measurements. The reported specific activity of the commercial [^{3}H]RAF was determined and found to be correct, and its purity was confirmed by paper chromatography. There was also no evidence of hydrolysis of [^{3}H]RAF to its component sugars during the incubation with galactose oxidase or during the coupling reaction. The discrepancy between anthrone and radioactivity measurements may be explained by a kinetic isotope effect resulting from tritiation at the C-6 position of galactose and preferential oxidation of the unlabeled RAF by galactose oxidase. Neither increasing the time of exposure of [^{3}H]RAF to galactose oxidase, nor increasing the amount of enzyme in the standard incubations affected the extent of coupling of radioactivity to protein. Since radioactivity was the only parameter which could be conveniently assessed in either *in vivo* experiments or when RAF was coupled to glycoproteins, the amount of RAF coupled was normally based on the specific activity of the [^{3}H]RAF used. Under the standard conditions described in Fig. 2, 30 to 38 nmol of RAF were routinely coupled/100 nmol of albumin.

Table I describes the effect of varying selected reaction parameters on the amount of RAF bound to protein. The amount of RAF attached to albumin did not increase when the $NaBH_3CN$ concentration was greater than 1 mg/ml (Experiment 2) but was decreased at lower concentrations (Experiments 3 and 4). In control experiments RAF was not coupled to protein unless $NaBH_3CN$ was included in incubation mixtures. The extent of coupling was not affected when the reaction was incubated at 37°C (Experiment 5) but was decreased approximately 65% at 4°C (Experiment 6). The proportion of RAF coupled to albumin did not increase in a linear fashion when the proportion of aldehyde in the reaction mixture was increased (Experiments 7 and 8), suggesting a limited number of kinetically accessible amino groups under the conditions used. Finally, under standard conditions a characteristic amount of RAF was bound to each of the proteins tested (Experiments 9 to 11). As seen in Table I, asialofetuin was a much poorer substrate for the coupling reaction than fetuin, itself; therefore, [^{3}H]RAF-asialofetuin was prepared by enzymatic desialylation of [^{3}H]RAF-fetuin. The differential reactivity of fetuin and asialofetuin in the coupling reaction may result from a conformational change in fetuin's structure upon removal of charged sialic acid residues. Alterations in other reaction parameters, particularly pH and time of incubation (26), can be expected to enhance the extent of RAF attached to proteins; in order to minimize structural alterations in proteins to be studied *in vivo* particularly gentle conditions for coupling were chosen in these experiments.

TABLE I

Effect of varying reaction conditions on the coupling of raffinose to protein

Experiment	Conditions[a]				Results[b]
	Temperature	[$NaBH_3CN$]	Raffinaldehyde/protein[c]	[Protein]	
	°C	mM			*mol raffinose/100 mol protein*
1[d]	25	17	1:1	0.1 mM albumin	33
2	25	34	1:1	0.1 mM albumin	33
3	25	8.5	1:1	0.1 mM albumin	20.5
4	25	1.7	1:1	0.1 mM albumin	10.9
5	37	17	1:1	0.1 mM albumin	36
6	4	17	1:1	0.1 mM albumin	17
7	25	17	10:1	0.01 mM albumin	100
8	25	17	30:1	0.01 mM albumin	210
9	25	17	1:1	0.2 mM fetuin	20
10	25	17	1:1	0.2 mM asialofetuin	1
11	25	17	1:1	0.2 mM RNase B	10

[a] All coupling reactions carried out in 1 ml of 0.1 N potassium phosphate buffer, pH 7.5, for 24 h with other parameters varied as indicated; 17 mM $NaBH_3CN$ = 1 mg/ml.

[b] Amount of raffinose coupled based on acid-precipitable radioactivity.

[c] Raffinaldehyde generated from 1 mM raffinose at pH 7.5, 37°C, using galactose oxidase and measured by the Nelson procedure as described under "Experimental Procedures."

[d] Conditions of this reaction defined as standard.

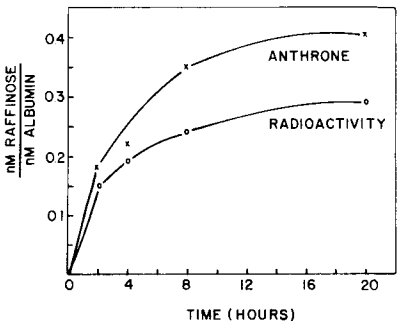

FIG. 2. Kinetics of coupling of raffinaldehyde to albumin. Equimolar (0.1 mM) amount of raffinaldehyde and albumin were incubated at pH 7.5 in the presence of 1 mg/ml of $NaBH_3CN$; at indicated times aliquots were removed and anthrone-positive or radioactive material precipitable by acid, or both, were quantitated as described under "Experimental Procedures."

In Vivo Experiments

Half-life Studies—Alterations in protein structure are often reflected by changes in their circulating half-life *in vivo* (2). Therefore, by comparing the half-lives of ^{125}I- and [^{3}H]RAF-labeled proteins *in vivo*, the effect of RAF attachment on this biological property of proteins can be evaluated. The results of studies comparing the kinetics of clearance of several ^{125}I- and [^{3}H]RAF-labeled proteins are shown in Fig. 3. Panel A demonstrates that, whether labeled with ^{125}I or [^{3}H]RAF, both asialofetuin and RNase B were rapidly cleared from the circulation. The rapid kinetics of clearance were expected since these proteins are recognized by separate carbohydrate-dependent clearance systems specific for glycoproteins with terminal galactose (asialofetuin (9)) and mannose (RNase B (11)) residues. The data of Panel A indicated that the attachment of RAF did not prolong the circulating half-life of asialofetuin and RNase B by interfering with these specific recognition systems.

In Fig. 3*B*, the circulating half-lives of ^{125}I- and [^{3}H]RAF derivatives of fetuin, a long lived protein, are compared. In this experiment fetuin was iodinated under mild conditions

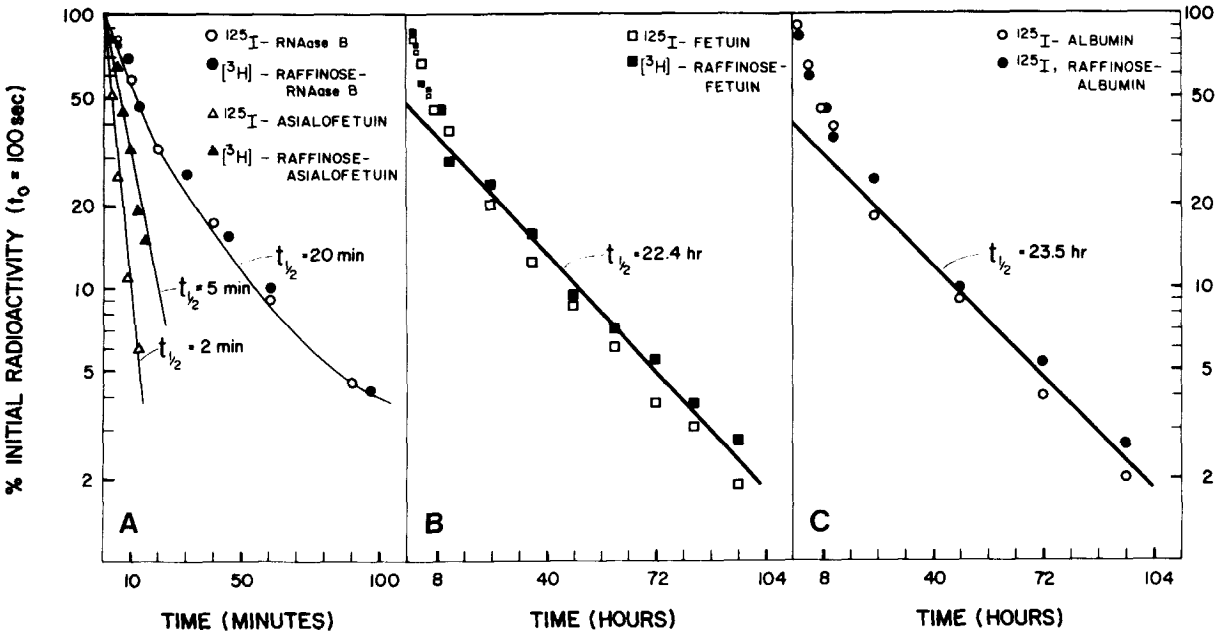

FIG. 3. Kinetics of clearance of various ^{125}I- and [^{3}H]RAF-labeled proteins from the rat circulation. Animals were injected with radiolabeled compound and blood samples were taken at indicated times; radioactivity was determined in measured plasma aliquots. Data is based on at least two animals/experiment and *points* represent average values, which typically varied by 5 percentage points. *Panel A*, △——△, 2 × 10^6 cpm (0.1 mg) of ^{125}I-asialofetuin; ▲——▲, 2.8 × 10^6 cpm (0.4 mg) of [^{3}H]RAF-asialofetuin; ○——○, 10 × 10^6 cpm (0.05 mg) of ^{125}I-RNase B; ●——●, 5 × 10^6 cpm (0.3 mg) of [^{3}H]RAF-RNase B. *Panel B*, □——□, 7 × 10^6 (0.1 mg) of ^{125}I-fetuin; ■——■, 5 × 10^5 cpm (0.5 mg) of [^{3}H]RAF-fetuin. *Panel C*, ○——○, 17 × 10^6 cpm (0.3 mg) of ^{125}I-albumin; ●——●, 17 × 10^6 cpm (0.3 mg) of ^{125}I (5 mol of raffinose/mol)-albumin.

using the lactoperoxidase technique (20) and then reisolated by gel chromatography so that minimal denaturation was expected. The chromatographic profile and half-life of [^{3}H]-RAF-fetuin were essentially identical to that of ^{125}I-fetuin, suggesting that the coupling of RAF did not cause significant changes in the physical or biological properties of the protein. As anticipated, the half-life of [^{3}H]RAF-fetuin, ~24 h, was in marked contrast to the 3- to 5-min half-life observed for the asialo derivative (9). Determination of the acid precipitability of ^{3}H radioactivity recovered from plasma over a 72-h period yielded 93 to 99% precipitability.

In experiments with bovine serum albumin (Fig. 3*C*), one preparation was labeled with ^{125}I, while another was first coupled with unlabeled RAF, in a molar ratio of 5:1 (RAF: albumin, determined by anthrone), and then iodinated by the lactoperoxidase technique (20). The circulating half-life of each preparation was ~24 h. In data not shown, the half-life of a commercial preparation of ^{125}I-human serum albumin was 17 h. These data suggest that modification of albumin with up to 5 molecules of RAF did not cause either an increased or decreased rate of uptake of albumin from the circulation.

Tissue Studies—In order to verify the stability of [^{3}H]RAF in tissue following protein uptake, the hepatic recovery of asialofetuin, heat-denatured albumin, and RNase B, labeled with either ^{125}I or [^{3}H]RAF, was compared. Table II illustrates that at early times postinjection the extent of recovery of radioactivity from proteins labeled by either method was comparable. However, for iodinated proteins, free iodide is rapidly released from the liver upon degradation of the protein (27). This loss is apparent even as early as 30 min, and by 24 h only traces of ^{125}I were recovered in liver from either ^{125}I-labeled asialofetuin or RNase B. In marked contrast, however, at 24 to 96 h, significant (90 to 60%) amounts of ^{3}H from [^{3}H]RAF/asialofetuin were still present in liver. Over a 96-h time period, ^{3}H radioactivity declined from 90% of dose at 30 min to 60% of dose at 96 h, giving an approximate half-life of 100 h. The recovery of radioactivity from liver at 24 and 48 h postinjection, 90 and 74%, was matched by a concomitant appearance of 14 and 26% of dose, respectively, in urine at these two times. Radioactivity in lung, kidneys, spleen, and heart at these same time points accounted *in toto* for less than 1% of dose. These data demonstrate that the radioactivity lost from liver following uptake of [^{3}H]RAF-asialofetuin was not reutilized by other tissues but was excreted in the urine. In contrast to the gradual decline of recovered radioactivity following asialofetuin injections, radioactivity from [^{3}H]RAF-heat-denatured albumin or RNase B appeared to be more stable over a 48- to 72-h period. RNase B experiments were limited to 48 h because the animals had been nephrectomized. Since asialofetuin is known to be cleared in hepatocytes (9), whereas RNase B and heat-denatured albumin are cleared in hepatic non-parenchymal cells (2, 6), differences observed in the stability of the RAF label from asialofetuin or heat-denatured albumin and RNase B injections may reflect differ-

TABLE II

Hepatic recovery of radioactivity from ^{125}I- and [^{3}H]raffinose-labeled proteins

Time postinjection	% injected dose[a]				Heat-denatured albumin
	Asialofetuin[b]		RNase B[b]		
	^{125}I	^{3}H	^{125}I	^{3}H	^{3}H[b]
h					
0.15	90				
0.5	58	90	20	22.5	38
24.0	<1	86	<1	26.5	32
48.0		74		19.0	
72.0		60			36
96.0	<1	60			

[a] Livers were removed at indicated times postinjection and homogenized, and radioactivity was determined in aliquots of homogenate as described under "Experimental Procedures." Results are the average value from at least two rats/time point which typically differed by less than 5 percentage points.

[b] Animals were injected with 2 to 5 × 10^6 cpm of ^{125}I- or [^{3}H]RAF-labeled proteins.

ences in the rate of turnover of cells or lysosomal contents in one cell type from another. In these experiments radioactivity not recovered in liver could be accounted for in blood and tissues of the reticuloendothelial system (see "Discussion"). The total body and hepatic distribution of radioactivity following injection of [^{3}H]RAF-RNase B was essentially identical to that previously reported for ^{125}I-RNase B (6).

Cell Separations—As a test of whether the RAF label would affect the known cellular sites of clearance of asialofetuin and heat-denatured albumin by parenchymal and nonparenchymal cells, respectively, the cell type in which radioactivity was localized after injection of each of these proteins was determined. Table III demonstrates that, based on the ratio of the specific activity (counts per min/cell) of the final purified parenchymal and non-parenchymal cell fractions, and an average ratio of two parenchymal cells/non-parenchymal cell in the initial cell suspension of liver (28), greater than 90% of the radioactivity from [^{3}H]RAF-asialofetuin injections was accounted for in hepatocytes. By a similar calculation, 85 to 90% of the hepatic radioactivity from heat-denatured albumin experiments was initially present in non-parenchymal cells. In experiments to be reported in detail elsewhere, [^{3}H]RAF-RNase B was also recovered primarily (>85%) in non-parenchymal cells at 0.5 to 24 h postinjection. There is some variation in relative specific activities of the two cell types among experiments, but this probably results from day to day variations in the cell isolation technique. The preferential localization of asialofetuin, heat-denatured albumin, and RNase B in the expected cell type supports the hypothesis that the attachment of RAF does not affect specific cellular uptake of proteins by pinocytic (asialofetuin, RNase B) or phagocytic (heat-denatured albumin) processes.

Subcellular Localization—The expected lysosomal localization of recovered radioactivity from [^{3}H]RAF-labeled proteins is illustrated in Table IV. The data also indicated that in addition to being stable in the liver for extended periods, the radioactivity from [^{3}H]RAF protein injections remained in the lysosomally enriched subcellular fraction over a 48- to 72-h period. The recovery of the lysosomal marker enzyme β-*N*-acetylhexosaminidase exactly paralleled that found for the radioactivity.

Characterization of Degradation Products—Fig. 4 shows

TABLE III

Hepatic cell type of recovery of radioactivity from [^{3}H]raffinose-labeled proteins

Time post-injection	[^{3}H]RAF-asialofetuin[a]		[^{3}H]RAF-heat-denatured albumin[a]	
	sp. act. PC[b] / sp. act. NPC	Calculated % cpm in PC[c]	sp. act. NPC[b] / sp. act. PC	Calculated % cpm in NPC[c]
h				
0.5	9.2	95	31	94
24.0			12.2	86
48.0	6.7	93		
72.0	12.1	96	11	85

[a] Animals were injected with 2 × 10^6 cpm of [^{3}H]RAF-asialofetuin or 4 × 10^6 cpm of [^{3}H]raffinose-heat-denatured albumin.

[b] The ratio of specific activity (sp. act.) is based on the counts per min/cell of the purified parenchymal (PC) and nonparenchymal (NPC) cells obtained from livers at indicated times after collagenase perfusion and differential centrifugation as described under "Experimental Procedures." Average purities of each cell type were 96 to 99% and average recoveries of parenchymal and non-parenchymal cells were 70 and 30% of cells in the initial cell suspension of liver, respectively.

[c] Calculated per cent of radioactivity in parenchymal or non-parenchymal cells in whole liver based on ratio of specific activities and observed average ratio of parenchymal/nonparenchymal cells of 2.

TABLE IV

Hepatic subcellular distribution of radioactivity recovered from [^{3}H]raffinose proteins

Time postinjection	% recovered radioactivity[a] in subcellular fraction					
	[^{3}H]RAF-asialofetuin[b]			[^{3}H]RAF-denatured albumin[b]		
	Nuclear	Mitochondrial lysosomal	Supernatant	Nuclear	Mitchondrial lysosomal	Supernatant
h						
0.5	13	83	4	20	70	10
24.0	17	74	9			
72.0				13	76	11

[a] Recoveries of radioactivity in fractions were 85 to 100% of that found in the whole homogenate.

[b] Animals injected with 2.5 × 10^6 cpm of [^{3}H]RAF-asialofetuin and 4 × 10^6 [^{3}H]RAF-heat-denatured albumin; livers were removed at indicated times and weighed portions homogenized in 0.25 M sucrose followed by differential centrifugation to obtain subcellular fractions described under "Experimental Procedures."

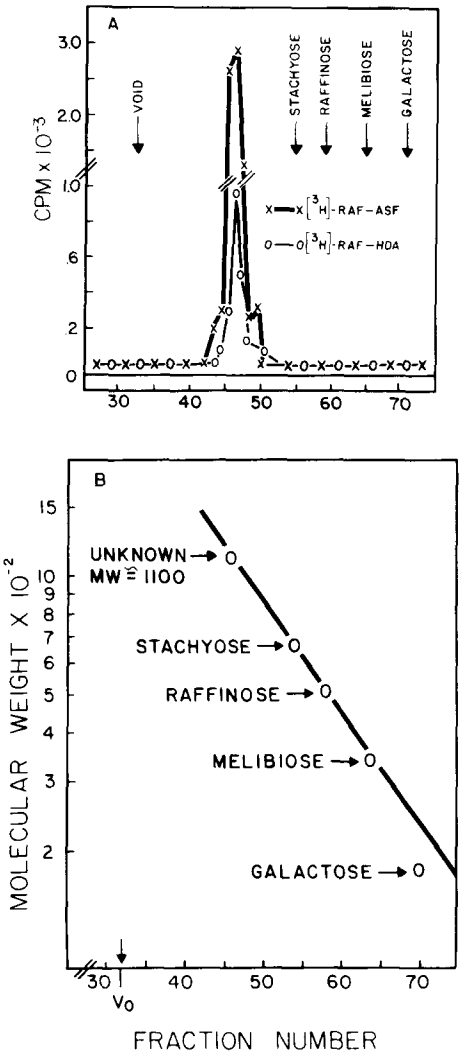

FIG. 4. Bio-Gel P-2 chromatography of soluble radioactivity recovered after administration of [^{3}H]RAF-proteins. *Panel A*, animals were injected with [^{3}H]RAF-asialofetuin ([^{3}*H*]*RAF-ASF*, ×——×) or [^{3}H]RAF-heat-denatured albumin ([^{3}*H*]*RAF-HDA*, O——O) and killed after 24 h, and soluble radioactivity was recovered after freeze-thaw of liver as described under "Experimental Procedures." Column dimensions, 110 × 1.5 cm; fraction size, 2.5 ml. *Panel B*, calibration of P-2 column with carbohydrate standards: stachyose (666), raffinose (504), melibiose (342), galactose (180) detected with anthrone.

the gel chromatographic profile of the soluble radioactivity recovered after freeze-thaw of livers obtained 24 h after administration of either [^{3}H]RAF-labeled asialofetuin or heat-denatured albumin. Essentially identical profiles were also found for degradation products obtained from livers at 72 and 96 h postinjection of [^{3}H]RAF-labeled heat-denatured albumin and asialofetuin, respectively. Based on the calibration curve shown in Fig. 4*B*, the molecular weight of the unknown peak was approximately 1100. Since essentially 100% of dose from asialofetuin injections was accounted for by the sum of recovered radioactivity in liver and urine, it seems likely that the entire RAF molecule is recovered attached to a small peptide derived from the protein. Paper chromatography of hydrolysates of the degradation products recovered from the P-2 column indicated that both radioactive fructose and glucose are recovered in a ratio essentially identical to that found for the starting protein.

DISCUSSION

A major gap in our knowledge of the metabolism of plasma proteins is the lack of essential information on the regulation of homeostatic concentrations of these proteins in plasma. To understand how plasma protein metabolism is regulated, it is at least necessary to identify the sites of catabolism of these proteins. The experiments described in this report represent an effort to overcome a major obstacle in the accurate determination of these sites of catabolism, namely that, although these proteins are delivered to tissues at a relatively slow rate (*e.g.* $t_{1/2} \geq 24$ h), rates of degradation in tissues (*e.g.* $t_{1/2} \leq 15$ min) are relatively rapid. Thus, any radioactive tracer attached to protein is lost from the site of uptake at a rate too great to allow quantitation.

We sought to develop an inert, indigestible radioactive tag which would accumulate at the tissue site of a protein's degradation and, thus, measure the cumulative degradation of the protein in that tissue. Sucrose seemed especially attractive as a radioactive ligand for this purpose because of its demonstrated stability in lysosomes. Recently, based on a similar rationale, Pittman and Steinberg (29) reported the attachment of [^{14}C]sucrose to low density lipoprotein and albumin, via a succinate spacer arm. Their *in vitro* studies of the uptake of the labeled proteins by cells in culture demonstrated both accumulation and stability of the radioactive label in cells over a 6-h period. While these initial observations are promising, there are limitations inherent in this method for attaching sucrose to protein. The attachment through the succinate spacer alters the charge of protein both by conversion of an amine to an amide function and by addition of negative charge from uncoupled succinate groups on the sucrose moiety. In addition, the ester linkage between sucrose and the linking succinate arm may well be susceptible to plasma esterases *in vivo*. In contrast, use of reductive amination to couple RAF, and thus sucrose, to protein results in minimal alterations in charge and isoelectric point of the modified protein. Further, we have observed that the label is completely stable under *in vivo* conditions. As noted above, the coupling procedure can be performed under mild, physiological conditions and does not result in any modification of gel chromatographic behavior of the proteins studied or the enzymatic activity of RNase B. The studies presented also document that the kinetics and sites of clearance of RAF-labeled proteins are essentially identical to those found for ^{125}I-labeled proteins, suggesting that, in fact, there is no major alteration in the physical and biological properties of the modified proteins.

A basic assumption in the choice of sucrose as in indigestible tracer is the absence of sucrase or β-fructofuranosidase activities in mammalian lysosomes. There has been, however, a report of a low but detectable level of sucrase in rat liver lysosomes (30). As shown above, while the RAF label is particularly stable in non-parenchymal cells, there was a measurable loss of the RAF radioactivity with time from hepatocytes. This could result from normal hepatocyte turnover but is unlikely to result from enzymatic degradation of the RAF-amino acid conjugate. As noted above, 100% of injected dose of [^{3}H]RAF-asialofetuin could be accounted for in liver and urine at all times. In addition, only a single low molecular weight species was detected on Bio-Gel P-2 from either of these sources, for as long as 96 h postinjection, and contained both glucose and fructose in the expected ratios. These data suggest that either RAF attached to protein is not a suitable substrate for the sucrase described by Horvat (30) or that the sucrase activity is too low to be detectable during the course of our experiments. It should also be noted that, since the loss of radioactivity from hepatocytes is not detectable for at least 24 h postinjection and has an approximate half-life of 100 h, experiments to study the sites of uptake of proteins with long circulating half-lives can be carried out over at least a 48-h period, with little loss of accumulating radioactivity in parenchymal cells, should this prove to be a site of uptake.

The emphasis of the experiments reported here has been to study the stability of the [^{3}H]RAF label in liver, as a model for its stability in other tissues. The data obtained to date verify this stability in liver and in the lysosomal subcellular fraction of both hepatic cell types. In preliminary studies, radioactivity not found in liver from [^{3}H]RAF-heat-denatured albumin injections could be accounted for primarily in kidney, spleen, and lung, other organs of the reticuloendothelial system expected to participate in the clearance of aggregated protein (2). The recovery of radioactivity in these organs appears to have a stability comparable to that found for liver, and only 6.5% of dose was found in urine over a 72-h period. The uptake of RNase B also occurs in organs of the reticuloendothelial system, but a large proportion is found in muscle and hide (6). Thus, this protein should also be useful for evaluating the stability of RAF in nonhepatic tissue.

Since the ultimate goal of our research is to investigate regulation of plasma protein catabolism, it was essential to document that the RAF label did not interfere with normal mechanisms of clearance or elicit clearance by different pathways. The recovery of asialofetuin in hepatocytes and RNase B and heat-denatured albumin in non-parenchymal cells of liver suggests that the normal mechanisms of clearance were operating on RAF-labeled proteins. The fact that ^{125}I- and RAF-labeled fetuin and albumin were cleared from the circulation with similar kinetics also suggests that attachment of RAF did not interfere with the normal uptake mechanisms for these proteins.

One other important aspect of the use of RAF as a label is that it should accumulate with time in the tissues active in catabolism of long lived proteins, as protein is slowly delivered to those tissues. In one set of preliminary experiments studying the hepatic recovery of [^{3}H]RAF-fetuin (circulating half-life, ~24 h), the recovery of radioactivity at 12, 24, and 48 h postinjection was 21, 29, and 36% of dose, respectively. At these same times there was a concomitant decrease in radioactivity in plasma from 41 to 23 to 12% of dose (Fig. 3). These encouraging initial findings with [^{3}H]RAF-fetuin are entirely consistent with the hypothesis that the recovery of the RAF label from a protein with a long plasma half-life would show a progressive increase in a tissue involved in the catabolism of that protein, as it is slowly removed from the circulation. An additional 7 and 14% of dose from [^{3}H]RAF-fetuin was re-

covered in urine at 24 and 48 h postinjection. Thus, blood, liver, and urine accounted for about 60% of dose at the three times studied. The remainder of radioactivity could be accounted for in other visceral organs and extravascular compartments. Details of these experiments will be presented elsewhere.

In summary, from the experiments presented above, we conclude that [^{3}H]RAF functions as a biologically inert, indigestible tracer which accumulates in tissues following protein removal from the circulation and intracellular degradation in lysosomes. We believe that the technology which we have developed here provides an exciting new tool for investigating the sites, and eventually, the regulation of plasma protein catabolism *in vivo*.

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