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John W. Baynes
University of South Carolina - Columbia, john.baynes@sc.edu

Finn Wold

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Effect of Glycosylation on the *in Vivo* Circulating Half-life of Ribonuclease*

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JOHN W. BAYNES‡ AND FINN WOLD§

From the Department of Laboratory Medicine and Pathology, School of Medicine, and the Department of Biochemistry, College of Biological Sciences, University of Minnesota, St. Paul, Minnesota 55108

The circulating half-lives of the four isozymes of bovine pancreatic ribonuclease (RNases A, B, C, and D) have been determined in normal and in nephrectomized rats. The isozymes differ only in their glycosyl content. While A contains no sugars, B has a simple oligosaccharide ($GlcNAc_2Man_{4-5}$), and C and D each have a complex oligosaccharide ($GlcNAc_4$ Man_{2-3} Gal_2 Fuc $NeuAc_2$, and $GlcNAc_4$ Man_3 Gal_2 Fuc $NeuAc_4$, respectively) attached to Asn-34 of the polypeptide chain. All four isozymes were cleared rapidly in normal rats ($t_{1/2}$ = 2 to 3 min), as expected on the basis of the established role of the kidneys in removing low molecular weight proteins from circulation. In nephrectomized rats, however, a much slower clearance was observed, thus permitting the evaluation of the role of the carbohydrate chains in the catabolism of the isozymes. The clearance curves can be analyzed in terms of two processes, a rapid initial one, shown to represent the equilibration of the injected enzyme into extravascular space, and a second one which is interpreted as the catabolic clearance of the enzyme. The half-life of the RNase isozymes was calculated from this second process and found to be in the range 528 to 577 min for RNase A, 15 min for RNase B, 681 to 862 min for RNase C, and 839 to 941 min for RNase D. The rapidly cleared RNase B was treated with α-mannosidase to remove 3 of the 4 mannosyl residues, leaving only a trisaccharide ($GlcNAc_2$-βMan) attached to the protein. The half-life of this RNase B derivative was found to be in the range 616 to 733 min. From these results it is concluded (*a*) that the addition of complex oligosaccharides to a protein does not have any significant direct effect on its circulating half-life (RNases C and D compared to RNase A), and (*b*) that in the rat there exists a mechanism for clearing glycoproteins based on specific recognition of exposed α-mannosyl residues (RNase B compared to the other isozymes and to α-mannosidase-treated RNase B).

The physiological sites and mechanisms involved in the homeostatic regulation of plasma protein turnover are as yet poorly understood. The role of prosthetic oligosaccharides as regulatory determinants for the catabolism of circulating glycoproteins has received much attention, however, since the pioneering investigations of Ashwell, Morell, and co-workers (2). They demonstrated that *in vitro* modifications of the oligosaccharides of several plasma glycoproteins can produce significant alterations in their circulating half-life, and have described a mechanism for the rapid hepatic catabolism of plasma asialo-(β-galactoside terminal)glycoproteins. More recent reports have indicated (3–5) that further degradation of plasma protein oligosaccharides, resulting in exposure of internal β-*N*-acetylglucosaminyl and core α-mannosyl residues, might also trigger the rapid clearance of these glycoproteins from the circulation. However, the extent to which these carbohydrate-specific clearance processes are involved in the *in vivo* regulation of plasma glycoprotein metabolsim is still unclear.

While the previous studies have focused on the effects of partially degraded oligosaccharides on the catabolism of glycoproteins, the goal of the present study was to assess the overall effect of the complete, native oligosaccharide component on glycoprotein metabolism. To achieve this goal we required a system in which the specific contribution of the oligosaccharide prosthetic group and the apoprotein component could be separately evaluated. Fortunately, nature has provided such a system, in the form of the four bovine pancreatic ribonuclease (RNase) isozymes. These isozymes, described by Plummer and Hirs (6, 7), consist of the same polypeptide chain, and differ only in their degree of glycosylation at asparagine residue 34: RNase[1] A is the unglycosylated "aglycone"; RNase B has the

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‡ Recipient of American Cancer Society Postdoctoral Fellowship PF-1147. Present address, Department of Chemistry, University of South Carolina, Columbia, South Carolina 29208.

§ To whom correspondence should be directed at Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108.

[1] The abbreviations used are: RNase, bovine pancreatic ribonuclease; DFP, diisopropyl phosphorofluoridate; Con A-agarose, insolubilized, agarose-bound concanavalin A; RNase B^c, RNase B isozyme specifically prepared from commercial RNase B (Sigma type

characteristic, simple plasma-type oligosaccharide side chain containing only mannose and *N*-acetylglucosamine; RNases C and D have complex plasma-type oligosaccharides containing galactose, fucose, and sialic acid, as well as mannose and *N*-acetylglucosamine. These isozymes of RNase are identical in amino acid composition, ultraviolet absorption spectra, and specific enzymatic activity, with tryptic peptide maps indicating that they have identical amino acid sequences (8). Thus, comparing the *in vivo* plasma half-lives of the various glycoprotein isozymes of RNase to the half-life of the aglycone RNase A it is possible to evaluate directly the effect of glycosylation on the plasma survival time of a polypeptide chain.

MATERIALS AND METHODS

Enzyme Assays—RNase activity was determined using either cytidine 2':3'-phosphate (9) or yeast RNA as substrate. Assays using RNA substrate were performed by a modification of the procedure of Anfinsen *et al.* (10). RNase (0 to 100 ng) was added to 0.05 ml of 0.1 M Tris·HCl, pH 7.4, which contained 1 M NaCl, and 0.2 mg/ml of bovine serum albumin to prevent adsorption of the enzyme on glass. The reaction was started by addition of 1 ml of 8 mg/ml of RNA in 0.1 M Tris·HCl, pH 7.4, incubated for 20 min at room temperature, and stopped by addition of 1 ml of 1.25% uranyl acetate in 12.5% trichloroacetic acid. Following centrifugation, 0.5 ml of supernatant was diluted to 2.5 ml with water, and the absorbance at 260 nm was determined. RNase A (Sigma, type 1A), purified by ion exchange on IRC-50 according to Hirs *et al.* (11), was used as the standard for estimation of RNase concentration.

Protein and Amino Acid Determinations—Protein concentration during RNase purification was estimated from absorbancy measurements, using an absorbance (280 nm) of 18 for a 1% solution of unfractionated pancreatic juice (12). Protein concentration in the purified RNase isozyme preparations was determined from amino acid analyses. Amino acid analyses were performed on the Beckman model 119 amino acid analyzer. Samples were adjusted to approximately 0.5 mg of protein/ml of 6 N HCl, and hydrolyzed in evacuated, sealed ampoules for 24 h at 110°.

Carbohydrate Analyses of RNase Isozymes—Neutral sugars were estimated on a Technicon carbohydrate analyzer according to Lee *et al.* (13, 14), following hydrolysis of 0.5 mg of protein/ml of 2 N trifluoroacetic acid for 4 h at 100°. Glucosamine was estimated with the amino acid analyzer following hydrolysis of 0.5 mg/ml of 3 N HCl for 9 h at 110°. Corrections for destruction of carbohydrates during hydrolysis were estimated from hydrolyses of known amounts of monosaccharides in the presence of RNase A. Sialic acid was determined by the thiobarbituric acid method of Warren (15) following hydrolysis of protein in 0.1 N H_2SO_4 for 1 h at 80°.

Disc Gel Electrophoresis—Electrophoresis of RNase isozymes was carried out at pH 4.0, in 15% cross-linked polyacrylamide gels for 6 h at 5 mA per gel according to McAllister *et al.* (16).

Purification of RNase Isozymes—Bovine pancreatic juice was obtained by pancreatic duct cannulation,[2] according to the method of Wass (17). Secretions were collected in iced containers changed at 12-h intervals, DFP (1 M in isopropanol) was added to a final concentration of 5×10^{-4} M, and the juice was lyophilized. The resulting powder was stored at −20°. RNase activity was found to be stable for at least 3 months under these conditions. All purification steps were carried out at 4°.

The lyophilized pancreatic juice powder was dissolved with stirring in distilled water (10%, w/v), and DFP was added to 1 mM final concentration. The solution was dialyzed in a Bio-Rad Bio-Fiber-50 beaker against 0.2 M sodium acetate buffer, pH 5.2, until chloride ion was no longer detectable in the dialysate by titration with 0.1 M $AgNO_3$. Precipitated protein was removed by centrifugation for 10 min at 12,000 × *g*. If necessary, the pH was adjusted to pH 5.2 with a few drops of 1 M acetic acid. This solution was then applied to the RNase affinity column prepared by coupling N^4-(6-aminohexyl)-cytidine 2'(3')-monophosphoric acid to activated CH-Sepharose 4B (Pharmacia) according to the procedure of Scofield *et al.*[3] RNase was applied at a level of 4 to 5 mg/ml of column volume, and was quantitatively adsorbed to the column under these conditions. The column was washed with 3 to 4 bed volumes of 0.2 M sodium acetate, pH 5.2, and then eluted with 4 M NaCl in the same buffer. More than 98% of the RNase was recovered in the eluate, resulting typically (see Fig. 1) in an 8 to 12-fold purification. At this point the RNase was approximately 25 to 30% pure. Elution of the affinity column with a gradient of NaCl in acetate buffer did not yield a significant improvement in purification.

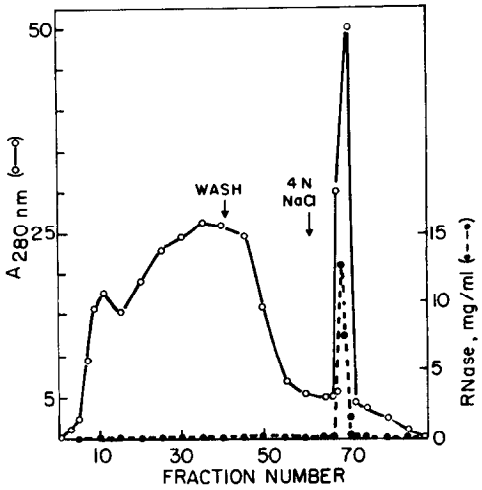

FIG. 1. Chromatography of pancreatic juice on RNase affinity column. Lyophilized pancreatic juice powder (16 g total weight, 6 g of protein), containing 170 mg of RNase activity in 280 ml of 0.2 *M* sodium acetate, pH 5.2, was chromatographed on a 40-ml (2 × 13 cm) RNase affinity column. Elution with 4 M NaCl resulted in recovery of 168 mg of RNase, with 11-fold purification. Fraction volume: 7 to 8 ml.

RNase-containing fractions were pooled and adjusted to pH 3.5 with 4 M HCl (7). After 1 h the mixture was centrifuged for 10 min at 12,000 × *g*; the precipitate was washed with 5 volumes of 0.2 M acetic acid and discarded. The pH 3.5 precipitation usually resulted in only about a 20% increase in purity with 90 to 95% yield. This step was included in the purification scheme, however, because it removed materials which otherwise precipitated during the subsequent dialysis and which tended to co-precipitate significant amounts of RNase.

The pH 3.5 supernatant and the acetic acid wash were pooled, and dialyzed in the Bio-Fiber device against 0.01 M Tris·HCl, pH 7.0. The progress of the dialysis was followed by monitoring the electrical conductivity of the dialysate. DFP was added to the dialysand (final concentration, 1 mM), and, if necessary, the pH was adjusted to 7.0 with a few drops of 1 M Tris base. In order to separate the RNase isozymes this solution was chromatographed on CM-cellulose (~500 mg RNase on a 2 × 12 cm column), using a 1-liter gradient of 0 to 0.15 M NaCl in 0.01 M Tris·HCl, pH 7.0. The elution profile of enzymatic activity on the CM-cellulose column was essentially identical to that shown in Ref. 7. Based on this elution profile the isozyme composition of the pancreatic juice was found to be 94% RNase A, 5% RNase B, 0.7% RNase C, and 0.5% RNase D.

The RNase-containing fractions corresponding to RNase B, and C + D were pooled from several column runs for further purification. Only RNases B, C, and D were purified from pancreatic juice. The fraction corresponding to RNase A was not processed further. The RNase A used as the enzyme standard and for injection experiments was the repurified commercial RNase A (Sigma type IA).

To remove contaminating RNase A, the pooled RNase B peak was chromatographed on a column of Con A-agarose (Sigma). The RNase A passed through the column unretarded while RNase B was bound selectively (*cf.* Fig. 2*a*), and was eluted with 10% α-methyl-D-glucopyranoside. The column capacity was approximately 1.3 mg of RNase B/ml of gel volume of a Con A-agarose gel rated at 3.7 mg of

XII-B); RNase B^x, the product of α-mannosidase digestion of RNase B^c.

[2] The authors wish to express their appreciation to Dr. D. E. Otterby and Mr. S. A. Nelson, of the Department of Animal Science, University of Minnesota for donating and caring for the animals used in this study, and to Dr. E. A. Usenik, Department of Surgery, University of Minnesota School of Veterinary Medicine, for performing the pancreatic duct cannulations.

[3] R. E. Scofield, R. P. Werner, and F. Wold, submitted for publication.

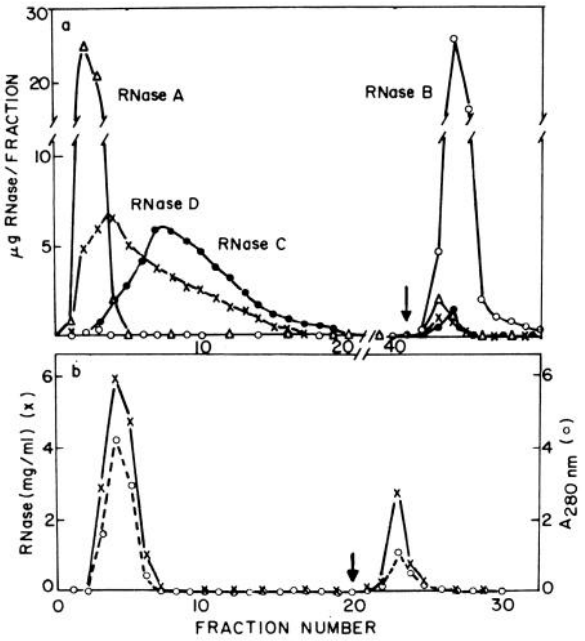

FIG. 2. Chromatography of RNase isozymes on Con A-agarose. *a*, samples of 50 μg of the purified RNase isozymes were dissolved in 250 μl of 0.1 N Tris·HCl, pH 7.4, containing 1 M NaCl, 1 mM $CaCl_2$, $MgCl_2$, and $MnCl_2$, and applied to a 1-ml Con A-agarose column prepared in a disposable pipette. The column was eluted at the rate of 1 ml (1 bed volume) per 10 min, and 1-ml fractions were collected. After 40 column volumes of the starting buffer, the column was eluted with 10% α-methyl-D-glucopyranoside in the same buffer. Elution profiles for RNases C and D varied with the amount of enzyme and rate of elution, but complete elution of these isozymes required 10 to 20 volumes of buffer. *b*, 100 mg of commercial RNase B (Sigma type XII-B) was dissolved in 10 ml of above buffer, and applied to a 15-ml (2 × 5 cm) Con A-agarose column. Fractions of 5 ml were collected; the *arrow* marks the beginning of elution with 10% α-methyl-D-glucopyranoside. Recovery was greater than 98%. On five separate applications 19 to 21% of RNase activity was eluted by α-methyl-D-glucopyranoside.

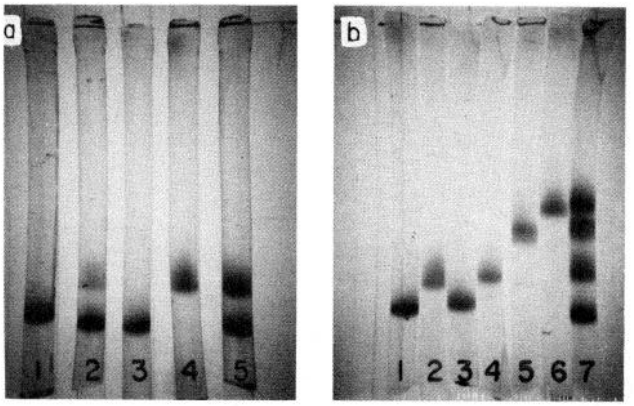

FIG. 3. Polyacrylamide disc gel electrophoresis of RNase isozymes. Protein samples (15 to 20 μg) were electrophoresed as described under "Materials and Methods." *a*, *Lane 1*, RNase A; *2*, Sigma type XII-B RNase B, showing approximately 4:1 ratio of two components; *3*, the component which is not retarded on Con A-agarose; *4*, RNase B^c, the component eluted from Con A-agarose with α-methyl-D-glucopyranoside; *5*, mixture of equal amounts of each component. *b*, *Lane 1*, RNase A; *2*, RNase B^c; *3*, RNase B^x; *4*, RNase B; *5*, RNase C; *6*, RNase D; *7*, a mixture of equal amounts of RNases A, B, C, and D.

TABLE I

Carbohydrate composition of ribonuclease isozymes

For comparable data, see Ref. 7.

Ribonuclease isozyme	A	B^c	B^x	B	C	D
	mol carbohydrate/mol protein					
Glucosamine	0	1.9	1.8	2.0	4.1	3.9
Mannose	0	4.7	1.0	4.3	2.6	2.9
Galactose	0	0	0	Tr[a]	2.0	1.9
Sialic acid	0	0	0	0	2.2	4.0
Fucose	0	Tr	0	Tr	1.0	1.1

[a] Tr, trace.

yeast mannan/ml. The RNase B pool from the lectin column was finally rechromatographed on the RNase affinity column as described previously. The pooled RNase C + D peak from the CM-cellulose column was also rechromatographed on the RNase affinity column. RNase C and D isozymes were then separated by chromatography on CM-cellulose in a 1-liter gradient of 0.02 to 0.075 M NaCl in 0.01 Tris·HCl, pH 7.0. RNase C was further purified by isolation of the fraction retarded on Con A-agarose (Fig. 2*a*).

Since the commercial product was a more convenient source of the RNase B isozyme, Sigma RNase B (type XII-B) was purified by chromatography on Con A-agarose as shown in Fig. 2*b*. The fraction which was bound by the lectin column and was eluted by α-methyl-D-glucopyranoside, accounted for about 20% of the commercial RNase B applied to the column, and was termed RNase B^c. The remaining 80% of the commercial RNase B was eluted in the void volume of the column, and did not contain carbohydrate. The heterogeneity of the commercial RNase B, and its separation into two components by chromatography on Con A-agarose is verified in the disc gel electrophoresis patterns shown in Fig. 3*a*.

Glycosidase Digestion of RNase B^c—Jack bean α-mannosidase, kindly provided by Dr. Gary Nelsestuen (University of Minnesota), had a specific activity of 30 μmol of *p*-nitrophenyl-α-D-mannopyranoside hydrolyzed per min at 37° per mg of protein. This enzyme had no detectable β-*N*-acetylglucosaminidase or β-mannosidase (<0.01%) activity using *p*-nitrophenylglycosides as substrates. α-Mannosidase (0.3 unit) was added to 12.5 mg of RNase B^c in 5 ml of 0.1 N sodium acetate, pH 5.0, containing 1 mM $ZnSO_4$ (37°). An additional 0.3 unit of α-mannosidase were added at 12 and 24 h. Aliquots (0.1 ml) were removed at various times, and assayed for mannose released by a coupled hexokinase/pyruvate kinase/lactate dehydrogenase system, according to Tarentino *et al.* (18). The mannose release leveled off after 36 h, at which time a total of 3.1 mol of mannose had been removed per mol of RNase B^c. At 36 h a sample of the digestion mixture was applied to the carbohydrate analyzer, and 3.0 mol of free mannose were detected per mol of RNase B^c. The product of this α-mannosidase digestion of RNase B^c was isolated by Sephadex G-75 chromatography and is referred to as RNase B^x. Carbohydrate analyses indicated that RNase B^x contained 2 *N*-acetylglucosamine and 1 mannose residue. Based on the structural analysis of Sukeno *et al.* (19), the residual mannose in β-linked to an *N*-acetylglucosamine residue, thus explaining its resistance to α-mannosidase.

Characterization of RNase Isozymes—Table I shows the carbohydrate composition of the RNase isozymes used in this study. These data are in good agreement with those previously published by Plummer and Hirs (6, 7). The homogeneity of the isozymes is supported by the electrophoretic patterns shown in Fig. 3*b* in which each isozyme yields a single band of staining material with a distinct electrophoretic mobility. RNase B, prepared from pancreatic juice, and RNase B^c, prepared from the commercial product, behave identically on disc gel electrophoresis, consistent with the similarity in their carbohydrate composition. When chromatographed on Sephadex G-75 each isozyme yielded a single peak of protein and enzymatic activity, eluting at a volume corresponding to the monomeric form of the enzyme; there was no evidence of aggregation. The relative specific activity of the various isozymes, using cytidine 2′:3′-phosphate as substrate, was: A = 100, B = 96, B^c = 96, B^x = 93, C = 94, D = 98. Using RNA as substrate, the relative specific activities were: A = 100, B = 84, B^c = 78, B^x = 80, C = 78, D = 77. From a comparison of the relative specific activities using the two different substrates, it appears that the oligosaccharide in the glycoprotein isozymes causes a slight inhibition of the activity of RNase toward the macromolecular RNA substrate.

Preparation and Sizing of Fluorescein-labeled Dextran—Dextran T-10 (Sigma) was reacted with fluorescein isothiocyanate according to

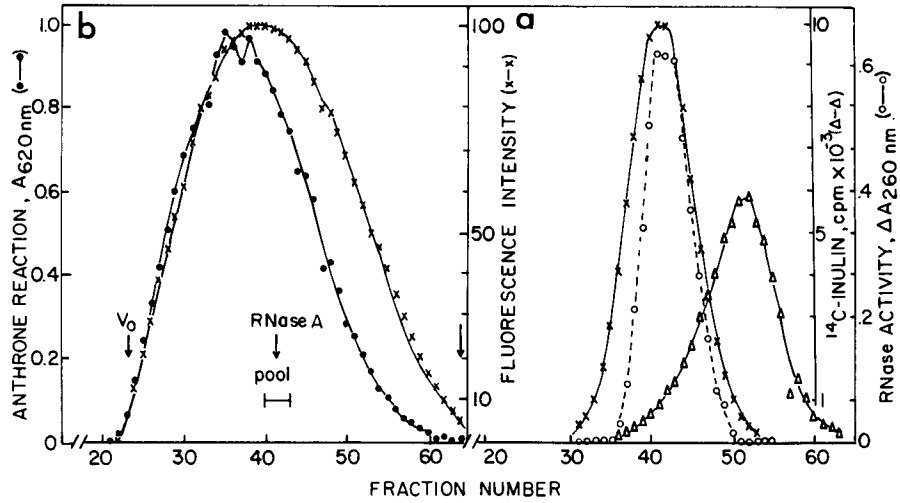

FIG. 4. Molecular sizing of fluorescein-labeled dextran, RNase, and inulin. *a*, fluorescein/dextran (100 mg) was dissolved in 1 ml of 0.2 M ammonium acetate and chromatographed on a column (1 × 35 cm) of Sephadex G-75. Fractions of 0.5 ml were collected. Aliquots were assayed for anthrone reactivity (21), and fluorescence at 518 nm (excitation 493 nm) on a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer. Fractions 40 to 43, corresponding in size to RNase A, were pooled. *b*, co-chromatography of pooled fluorescein/dextran (0.1 mg), RNase A (0.1 mg), and [^{14}C]inulin (0.1 μCi) under above conditions. Aliquots of each fraction were assayed for fluorescence, enzyme activity, and radioactivity.

the method of deBelder and Granath (20), using dibutyltin dilaurate (Ventron/Alfa) as catalyst. The product was washed three times by precipitation with ethanol, then dissolved in distilled water (5% w/v) and dialyzed overnight against 100 volumes of distilled water. The dialysand was lyophilized, redissolved in 0.2 M ammonium acetate (5% w/v), and chromatographed on a calibrated Sephadex G-75 column in the same buffer (Fig. 4*a*). Fractions 40 to 43, corresponding to the elution volume of RNase A, were pooled and lyophilized. A mixture of RNase A and the sized dextran derivative was chromatographed on the same G-75 column to show that the collected fraction of dextran was similar in molecular size to RNase A (Fig. 4*b*). In addition, [^{14}C]inulin (New England Nuclear, Inulin-carboxyl, [*carboxyl*-^{14}C]inulin, 19.3 mCi/g), M_r = 5500, was also chromatographed in the same column run to verify its molecular size and the absence of low molecular weight degradation products; the data for the [^{14}C]inulin is shown here since the [^{14}C]inulin and fluorescein/dextran are used later in the same experiment (Fig. 7).

Animal Experiments—Experiments were performed using normal or nephrectomized male albino rats weighing 300 to 360 g. Bilateral nephrectomies were performed under ether anesthesia through a midline incision. The renal vein and artery, and the ureter were ligated, and the kidneys were excised. The incision was sutured, and the animal was allowed to recover for 6 to 12 h. Ribonuclease isozymes (500 μg) were injected intracardially in 100 to 500 μl of physiological saline. Blood samples (75 μl) were obtained at timed intervals from lateral tail veins. The first sample was generally taken at 100 s after injection. Blood was collected in heparin-treated capillary pipettes, and plasma was separated by centrifugation. Aliquots of plasma (3 μl) were assayed in duplicate for RNase activity. Endogenous activity in both normal and nephrectomized rats was 0.6 ± 0.2 μg of RNase/ml of plasma.

RESULTS

RNase A, RNase B, and a 1:1 mixture of RNase C and D were injected separately into normal rats. The data in Fig. 5 show that all of the isozymes are rapidly cleared from the plasma with half-lives of 2 to 3 min. These results confirm previous reports on the rapid clearance of RNase A in the rat (22–24), and also establish that the carbohydrate prosthetic groups on RNases B, C, and D do not significantly alter the rate of clearance of these isozymes in the normal rat.

The site of RNase clearance has been localized primarily in the kidney (22–26), and reflects the major role for this organ in the catabolism of proteins of molecular weight lower than

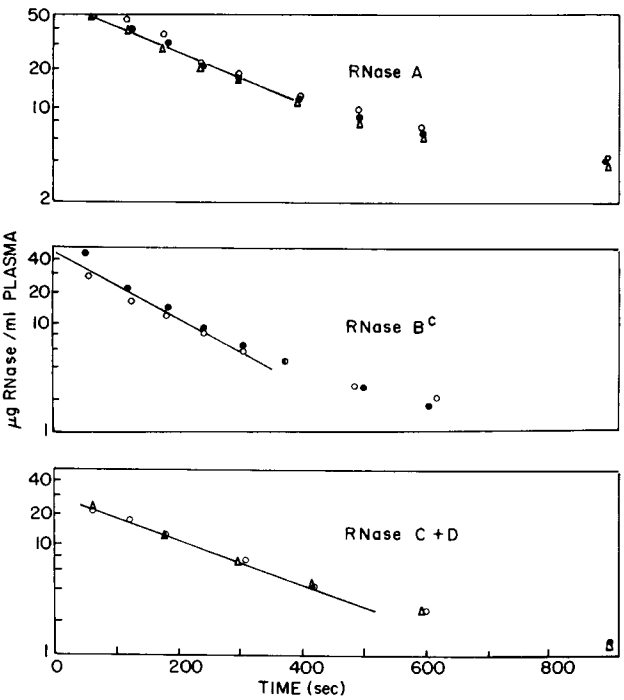

FIG. 5. Clearance of RNase isozymes from plasma of normal rats. RNase isozymes (500 μg) were injected into weight-matched groups of two or three rats. Half-lives are calculated from least squares fit through all *points* on *lines* drawn in figure. *a*, RNase A, $t_{1/2}$ = 155 s; *b*, RNase B^c, $t_{1/2}$ = 132 s; *c*, mixture of RNase C + D (1:1), $t_{1/2}$ = 149 s.

albumin (27, 28) which can readily permeate the glomerular basement membrane. Because the rapid, nonspecific renal clearance of RNase could prevent the observation of other clearance mechanisms operating specifically on the plasma proteins, all subsequent experiments were performed with nephrectomized rats.

Fig. 6 shows the results of injection of RNase A into nephrectomized rats. The clearance curve is complex, consist-

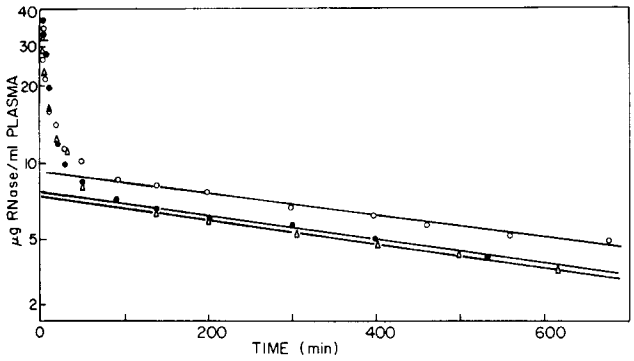

FIG. 6. Clearance of RNase A from plasma of nephrectomized rats. RNase A (500 μg) was injected into three rats, and half-lives were estimated as 528 (●), 537 (Δ), and 577 (○) min, respectively, from the least squares fit through *points* at times greater than 100 min.

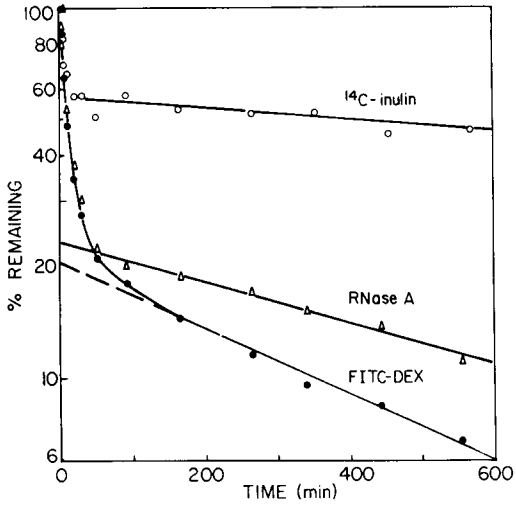

FIG. 7. Comparison of clearance kinetics for RNase A, fluorescein-labeled dextran (*FITC*), and [^{14}C]inulin. A mixture of RNase A (500 μg), dextran (2 mg), and [^{14}C]inulin (10 μCi) in 0.4 ml of physiological saline was injected into a 350-g nephrectomized rat. Data is shown as per cent of 100-s time point which contained 34.4 μg of RNase/ml, and 1550 cpm of ^{14}C per 10 μl. The C_2 intercept on the inulin curve is higher than the RNase and dextran *curves*, reflecting the more rapid rate of inulin ($M_r \sim 5500$) equilibration into extravascular compartments. The C_1 and C_2 intercepts for RNase A were at 105 and 23%, respectively, and for dextran, 109 and 21%. The half-life of RNase A in this experiment was 5.6 and 540 min for the first and second phases, respectively, and, for fluorescein-labeled dextran, 5.0 and 360 min.

ing of a rapid, initial decline, followed by a slower first order clearance process, as is frequently observed in enzyme injection experiments (29, 30). The first phase is generally attributed to diffusion and equilibration of the enzyme into extravascular compartments, and the second, slower phase results from catabolic clearance of the enzyme from the circulation. A least squares fit was calculated for the second phase of the decay curve, and the slope, b_2, and intercept, C_2, were calculated. The half-life of RNase A, ($t_{1/2} = 0.693/b_2$), was found to be 528, 537, and 577 min in three injection experiments.

Several lines of evidence suggest that the initial rapid decline in plasma RNase resulted from an equilibration process rather than from a selective removal of a unique fraction of the injected enzyme. Thus, no heterogeneity was detected in any of the RNase samples by ion exchange chromatography on CM-cellulose or IRC-50, by gel permeation on Sephadex G-75 (Fig. 4), or by gel electrophoresis (Fig. 3). In addition, efforts to detect a binding protein which might mediate the removal of a fraction of the RNase as a complex were unsuccessful. No inhibition of RNase activity was observed upon mixing with plasma, nor was the elution volume of RNase on Sephadex G-75 altered after incubation with plasma.

In a more direct experiment to establish that the first phase in the plasma decay curve resulted from an equilibration process, the fluorescein-labeled dextran with approximately the same molecular size as RNase (Fig. 4) was injected simultaneously with RNase A. As shown in Fig. 7 the kinetics of the initial clearance of RNase A and the dextran derivative are essentially identical, consistent with the hypothesis that molecules of the same size should diffuse at identical rates into extravascular compartments. The difference in the kinetics of clearance during the second phase can be attributed to differential rates of catabolism of the RNase A and dextran.

Analysis of the decay curves shown in Fig. 7 permits an estimate of the relative and absolute sizes of the plasma and extravascular compartments. By the process of "curve peeling" (29) the decay curves were described by the sum of two exponential processes, *e.g.*

$$[\text{RNase}] = C_1e^{-b_1t} + C_2e^{-b_2t}$$

where [RNase] is the plasma RNase concentration; and C_1 and C_2 are the intercepts, and b_1 and b_2 the slopes of the rapid and slow processes, respectively. According to Sterling (29, 31) the intercept, C_2, can be considered to represent the hypothetical concentration of the substance at zero time after equilibration with extravascular space (EVS), and the intercept, C_1, to represent the concentration of a substance that would have existed in the plasma volume (Pl) at zero time if no equilibration had taken place. Then, the approximate ratio of compartment sizes can be estimated from the equation:

$$\frac{C_1}{C_2} = \frac{\text{EVS}}{\text{Pl}}$$

Computed in this manner, the ratio of compartment sizes (EVS/Pl) for RNase A is 4.6, and for the sized dextran, 5.2 (data in legend to Fig. 7). From the similarities in the kinetics of clearance, and the $C_1$and C_2 intercepts for the RNase and dextran curves, it appears that these molecules are equilibrating not only at the same rate but also in the same ratio into plasma and extravascular compartments. From the data in this and the other experiments (*cf.* Figs. 6 and 8), the extravascular compartment appears to be about 4.5 to 5.5 times as large as the plasma compartment for a molecule of the size of RNase A.

The total volume, V_T, into which the injected material is distributed can be independently estimated from the quantity injected (Q) and the concentration at C_2 (after equilibration, but before clearance), *i.e.*

$$\frac{Q}{C_2} = V_T = \text{Pl} + \text{EVS}.$$

In the experiment in Fig. 7, the injection of 500 μg of RNase produced a C_2 intercept of 7.5 μg of RNase/ml of plasma (*cf.* Fig. 7 legend), which yields a total distribution volume, V_T = 67 ml for RNase A. An independent estimate of V_T was obtained by simultaneously injecting 10 μCi of [^{14}C]inulin, resulting in V_T = 63 ml for inulin. The estimated V_T for dextran was 49 ml, but this is probably the least accurate V_T estimate since serum proteins altered the dextran's fluorescence and thus introduced uncertainties in the estimates of Q. The data obtained with RNase A and [^{14}C]inulin in a 350-g rat,

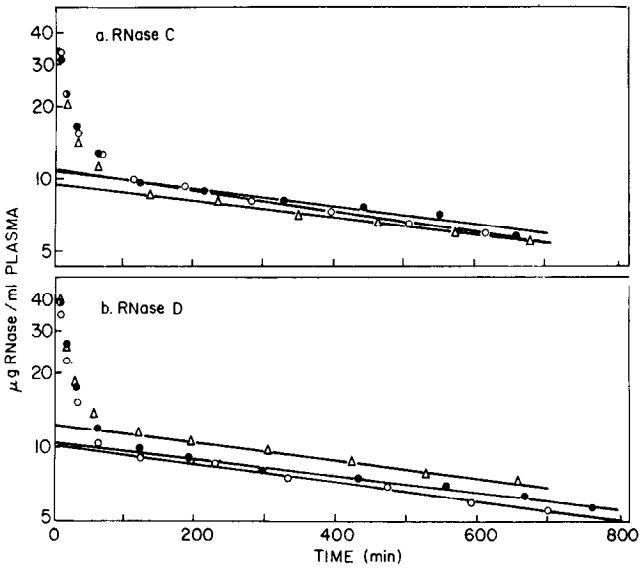

FIG. 8. Clearance of RNases C and D from plasma of nephrectomized rats. *a*, RNase C (500 μg) injections into three rats resulted in estimated half-lives of 681 (○), 830 (△), and 862 (●) min. *b*, RNase D (500 μg) injections yielded half-lives of 839 (○), 859 (△), and 941 (●) min.

indicate that the extracellular fluid volume is about 16 to 20% of the body weight. These estimates are consistent with the estimated plasma volume of 3% of the body weight in the rat (32) and an extravascular compartment of about 5 times the plasma volume. These volumes and ratios may not, however, represent the normal physiological situation in the rat since the animals used in these experiments had been nephrectomized 6 to 12 h earlier.

Having established that the initial rapid clearance of RNase from plasma is an equilibration process, our attention was now focused on the second, slower phase which represents the catabolic clearance of the molecule. Fig. 8, *a* and *b*, shows the plasma decay curves for injections of RNases C and D. The half-lives for RNase C in three rats were 681, 830, and 862 min, and for RNase D in three rats 839, 859, and 941 min. In three other preliminary experiments in which a partially purified mixture of RNase C and D (pooled C + D peak from first CM-cellulose fractionation) were injected, the half-lives of the C + D mixture were 691, 787 and 799 min. In all experiments the half-lives of the RNase isozymes containing complex carbohydrates (C and D) were 25 to 75% longer than the half-life of RNase A. The half-lives of RNases C and D were not significantly different from one another.

Fig. 9 shows that, in contrast to RNases A, C, and D, RNase B and RNase B^c are rapidly cleared from the circulation with a half-life of approximately 15 min. At 3 h after the RNase B and B^c injections the residual plasma RNase activity was generally indistinguishable from endogeneous levels, while levels of RNase A, C, and D at this time consistently were at 5 to 10 times the endogenous activity.

Since the only difference between RNase B or B^c and the other RNase isozymes is the presence of the simple oligosaccharide at Asn-34, it is reasonable to propose that this oligosaccharide, and specifically its terminal α-mannosyl residues, might be involved in the rapid clearance of RNase B. To test this proposal RNase B^c was treated with jack bean α-mannosidase (see "Materials and Methods") resulting in the release of all but 1 mannose residue per RNase molecule. The

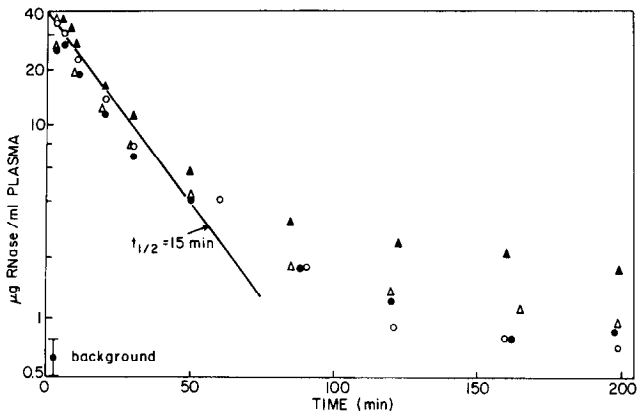

FIG. 9. Clearance of RNase B and B^c from plasma of nephrectomized rats. Two animals were used for each isozyme (500 μg of RNase per injection). *Black bar* at *lower left* shows the range of background activity for these animals. The *line* with $t_{1/2}$ = 15 min is a least squares fit through all *points* at less than 60 min, and is drawn as a convenient reference only; the slope of the clearance curve appears, in fact, to be varying continuously during the experiment.

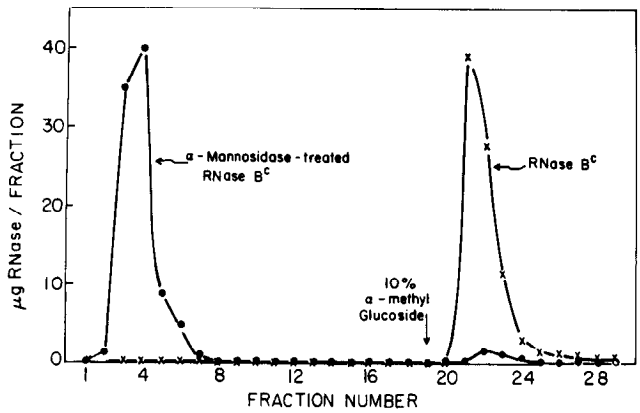

FIG. 10. Chromatography of RNase B^c on Con A-agarose before and after α-mannosidase treatment. RNase B^c was treated with α-mannosidase as described under "Materials and Methods." Aliquots (100 μg) of the original RNase B^c, and the product, RNase B^x, were chromatographed on 1-ml columns of Con A-agarose as described in Fig. 2*a*.

product of the mannosidase digestion, RNase B^x, was reisolated by chromatography on Sephadex G-75. The purified RNase B^x was no longer bound by concanavalin A (Fig. 10); this is consistent with the fact that its α-mannosyl residues, the recognition site for binding to concanavalin A, had been enzymatically removed. Chemical analysis of RNase B^x suggests that its core trisaccharide (19), Asn-$(GlcNAc)_2 \overset{\beta}{\leftarrow} (Man)_1$, is still intact.

Fig. 11 depicts the results of injection experiments with RNase B^x. The rapid clearance characteristic of RNase B^c was not observed after removal of the α-mannosyl residues of the simple oligosaccharide. The half-life of RNase B^x in three rats was 616, 636, and 733 min, an average of about 20% longer than that of RNase A.

DISCUSSION

Since the several isozymes of RNase differ only in their carbohydrate prosthetic group, the differences in the plasma half-lives of these isozymes can be attributed to the influence

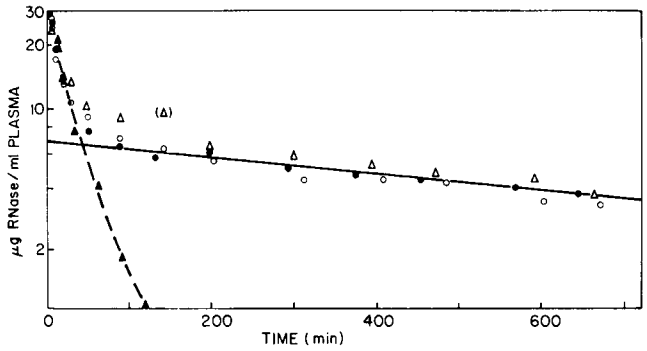

FIG. 11. Clearance of RNase B^x from plasma of nephrectomized rats. Three animals were injected with 500 μg of RNase B^x, resulting in estimated half-lives of 616 (Δ), 636 (○), and 733 (●) min. The kinetics of clearance of the original RNase B^c is shown (▲- - -▲) for comparison.

TABLE II

Plasma half-lives of RNase isozymes

RNase Isozyme	Measured half-life
	min
RNase A	528, 537, 540, 577
RNase B	~15
RNase B^c	~15
RNase B^x	616, 636, 733
RNase C	681, 830, 862
RNase D	839, 859, 941

of the specific type of oligosaccharide moiety in the isozyme. The findings from the comparison of the half-life of the isozymes are summarized in Table II and include two significant, new observations concerning the effect of glycosylation on the plasma survival time of a protein. First, in comparison to the nonglycosylated protein the attachment of a complex, acidic oligosaccharide to the polypeptide chain results in only a relatively small ($\leq$75%) increase in the half-life of RNase in the circulation. Secondly, the attachment of a simple, neutral, α-mannosyl-terminal oligosaccharide at the same site on the same polypeptide chain results in a significant, more than 30-fold decrease in its half-life in the circulation.

It seems unlikely that the major biological role for the complex oligosaccharides is reflected in the slight increase in half-life of RNases C and D as compared to RNase A. Indeed, the increase in half-life of RNases C and D might be attributed solely to the shift in the isoelectric point (pI) of RNase produced by the 2 or 4 sialic acid residues at the end of the oligosaccharide chain. Rutter and Wade (33) and Holcenberg *et al.* (34) have shown that the half-life of an enzyme in plasma is closely related to its pI. Thus, one would predict that the lowered pI of RNases C and D relative to RNase A would be adequate to explain their increased plasma half-life.

We consequently conclude that the intact complex oligosaccharide of the plasma glycoproteins may not directly affect the rate of these protein's catabolism in the circulation relative to the corresponding nonglycosylated proteins. However, the previous work which has clearly established hepatic removal of asialo-(β-galactosyl-terminal)glycoproteins from the mammalian circulation (2), and asialo-agalacto-(β-N-acetylglucosaminyl-terminal)glycoproteins from the avian circulation (3), leaves little doubt that the partially degraded, or incompletely synthesized oligosaccharide carries important messages. The messages in the cases studied to date appear to function in restricting the site of catabolism of certain glycoproteins to the liver. These proteins may normally be present in only trace amounts in plasma, and Burger *et al.* (35, 36) have, for example, presented evidence which indicates that the catabolism of transcobalamin II specifically in the liver via the Ashwell-Morell pathway might be involved in the regulation of vitamin B_{12} homeostasis. At this stage it appears, therefore, that the intact complex carbohydrate chain, which may not itself affect the half-life of circulating glycoproteins, carries a potential tissue-directing signal, which must be uncovered by alteration in its covalent structure before it can be read.

The results presented in this paper demonstrate that an additional "hidden" signal in the RNase isozymes is specifically expressed by exposed, nonreducing terminal α-mannosyl residues. When RNase A has been biologically modified by the attachment of a simple oligosaccharide to form RNase B, its half-life in the circulation was found to be reduced by about 30-fold. This change could be reversed by enzymatic removal of the α-mannosyl residues of RNase B, and the product, RNase B^x, returned to a half-life comparable to that of RNase A. These observations clearly support the existence of a carbohydrate-specific clearance process in the rat, which recognizes glycoproteins with terminal α-mannosyl residues.

The mechanism of this α-mannosyl-dependent clearance process has not yet been determined, but the existence of a plasma-binding protein which could mediate the specific clearance of RNase B has been excluded. The presence of such a protein was considered unlikely in view of the rapid and essentially identical clearance rates for all the RNase isozymes through the kidney of the normal (un-nephrectomized) rat (Fig. 5). In addition, as mentioned previously, when RNase B was mixed with serum or plasma, a binding protein was not detectable through any inhibition of RNase activity on its high molecular weight (yeast RNA) substrate; nor was the elution volume of RNase B on Sephadex G-75 altered upon mixing with serum or plasma. Thus it seems that, by analogy to the clearance process described by Ashwell and Morell (2), the clearance of RNase B probably occurs by way of a membrane-binding protein, specific for the α-mannosyl residues of the simple oligosaccharide; and the list of carbohydrate-binding proteins involved in these recognition processes in vertebrates must be expanded from currently established β-galactosyl- (2) and β-*N*-acetylglucosaminyl-binding proteins (3) to include an α-mannosyl-binding protein as well.

The physiological function of this protein clearance mechanism is not known. It is interesting, however, to note that the simple oligosaccharide, containing only mannose and *N*-acetylglucosamine, is not a common component of plasma glycoproteins. It is found in IgM (37) and IgE (38), and possibly in IgA (39–41), but is apparently not present in IgG (42), α_1-acid glycoprotein (43), α_1-antitrypsin (44), ceruloplasmin (45), transferrin (46), or fetuin (47). Detailed information on oligosaccharide structures is unfortunately not available for the majority of the plasma proteins (*e.g.* ligand-binding proteins, clotting factors, and complement proteins) so that a true estimate of the frequency or distribution of the simple oligosaccharide structures cannot be made. For the immunoglobulins, IgM and IgE, the simple oligosaccharide may perform an important function. The fact that the immunoglobulins are relatively stable proteins in plasma suggests that the simple oligosaccharides in these proteins are normally sterically protected from the sensing mechanism which rapidly clears RNase B. (Perhaps the steric restriction explains why the complex oligosaccharide was not synthesized on that site.) It is

possible, then, that upon interaction with antigen, a conformational change in the antibody molecule could present the simple oligosaccharide to the environment, resulting in the clearance of the antibody·antigen complex. Such a process might be physiologically important, especially under conditions of antigen or antibody excess when macromolecular aggregates may not be formed, *e.g.* during the initial or final stages of an immune response. It is perhaps not a coincidence that, in both IgM and IgE, the simple oligosaccharides are located at homologous sites in the Fc region of the heavy chain, the region which is known to be involved in the disposition of antibody subsequent to antigen binding. Other ligand-binding proteins in plasma, which are rapidly cleared following complex formation, may also have simple oligosaccharides. Thus, for example hemoglobin·haptoglobin (48, 49), heme·hemopexin (49), and protease·α_2-macroglobulin (50, 51) complexes might be cleared in a manner similar to that proposed above for some antigen·antibody complexes.

The lysosomal glycosidases comprise a group of glycoproteins which are known to be rapidly cleared from the circulation following intravenous injection (52–54). And, while the details of the carbohydrate structures of these enzymes are not known, the limited information on their binding to insolubilized concanavalin A (55, 56) and their high mannose content (57, 58) suggest that the removal of these enzymes from the circulation may be mediated by the α-mannoside-dependent clearance process which we have described for RNase B. In this respect, Stahl *et al.* (54) have reported that the clearance of β-glucuronidase is apparently not mediated by liver receptors for asialo-(galactosyl-terminal)glycoproteins, since asialofetuin and asialo-orosomucoid failed to competitively inhibit the clearance of β-glucuronidase from the rat circulation. Thus it appears that, among other possibilities, the α-mannoside-dependent clearance process may be involved in regulating (*i.e.* limiting) the level of lysosomal enzymes in the circulation, or may participate in the transport of lysosomal enzymes or other proteins between cells and organs within the body.

In the above discussion, it has been tacitly assumed that the process in which the α-mannosyl residues of RNase B are recognized is directly exposed to the plasma compartment, and that the removal of RNase B takes place directly from that compartment. However, this is clearly not a necessary assumption in view of the rapid equilibration of the RNase into the extravascular compartment. Indeed, the mannose recognition and removal could occur equally well in either or both compartments. Future studies must be directed at establishing the organ specificity, the compartment location, and the physiological function of this clearance process.

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