HEPATIC UPTAKE OF PROTEINS COUPLED TO FETUIN GLYCOPEPTIDE*

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SUMMARY:- The glycopeptides of fetuin and γ G immunoglobulin have been covalently linked to lysozyme and albumin with the coupling reagent toluene 2,4 diisocyanate. When the fetuin glycopeptide-protein compounds were treated with neuraminidase to remove sialic acid, and then injected into rats, the coupled compounds were rapidly and selectively removed from the circulation by the liver, most likely by the hepatocytes. Removal of the exposed galactose residues of the asialofetuin-proteins with β-galactosidase prevented this clearance. Neither the native proteins nor the γ G glycopeptide-proteins are taken up by the liver, demonstrating the specificity of the phenomenon. Once the asialofetuin-protein complexes enter the hepatocytes, they are degraded within two hours.

Morell et al. have demonstrated that hepatocytes are able to recognize and remove from the circulation specific oligosaccharides containing galactose residues at their nonreducing terminus (1). The hepatocyte receptors interact with the appropriate oligosaccharide regardless of whether it is part of an intact glycoprotein or in the form of an isolated glycopeptide (1). These observations suggested to us a mechanism for selectively directing the uptake of specific molecules by the liver. By coupling the proper glycopeptide to a test molecule it should be possible to cause the molecule to be delivered selectively to hepatocytes. In this report we describe experiments which demonstrate that the asialoglycopeptide of fetuin covalently linked to lysozyme or albumin causes these proteins to be selectively taken up by the liver.

Materials and Methods:- The glycopeptide of fetuin (obtained from Grand Island Biological Co.) was prepared by the method of Spiro (2) and purified by gel filtration on Sephadex G-25 and Bio-Gel P-10 followed by paper chromatography using as a solvent butanol:acetic acid:water (12:3:5). The γG immunoglobulin glycopeptide was prepared as previously described (3).

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To couple the glycopeptide to the protein, a modification of the method of Schick and Singer (4) was used: To 2 μmoles of glycopeptide in 1 ml of 0.1 M phosphate buffer pH 7.5 at 4° C, a total of 0.1 ml of toluene 2,4 diisocyanate (Polysciences, Inc.) was added in four separate aliquots, with stirring, over a 60-minute period. The reaction mixture was then centrifuged at 12,000 x g for 30 minutes in a refrigerated Sorvall centrifuge and the supernatant fluid allowed to stand for another hour at 4°C before it was applied to a Sephadex G-25 column (19 x 0.9 cm) to separate the glycopeptide fraction (in the excluded volume) from the unreacted coupling reagent. The column was eluted with water, and the glycopeptide fraction was taken to dryness using a rotary evaporator. To the glycopeptide was added 1.0 ml of a solution of 0.1 M Borax pH 9.4 containing 2 μmoles of either lysozyme or guanidinated lysozyme (5) or 0.5 μmole of bovine serum albumin. Each of the proteins was iodinated

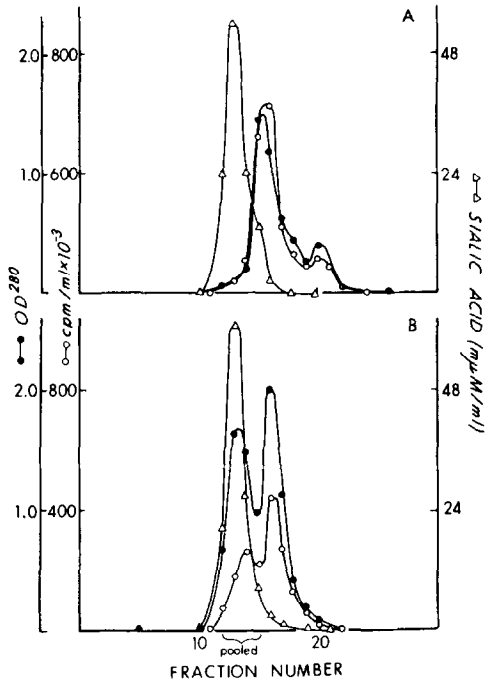

Fig. 1. Chromatography of lysozyme on Bio-Rex-70. A. 2 μmoles of ^{131}I-lysozyme were mixed with 2 μmoles of fetuin glycopeptide in the absence of coupling reagent and applied to the column. B. ^{131}I-lysozyme was coupled to fetuin glycopeptide as described under "Methods" and then applied to the column. Fractions of 1.6 ml were collected. ●—●, $O.D._{280}$; Δ—Δ, sialic acid; O—O, radioactivity.

by the chloramine-T method (6) prior to coupling with the glycopeptide. This reaction mixture was then incubated overnight at 37° under a toluene atmosphere

The glycopeptide-lysozyme reaction mixture was applied to a column of Bio-Rex 70 (40 x 0.9 cm) in 0.2 M phosphate buffer pH 7.5 - 0.25 M NaCl equilibrated by the method of Hirs (7). Under these conditions the coupled glycopeptide-lysozyme and the free glycopeptide were eluted before the uncoupled

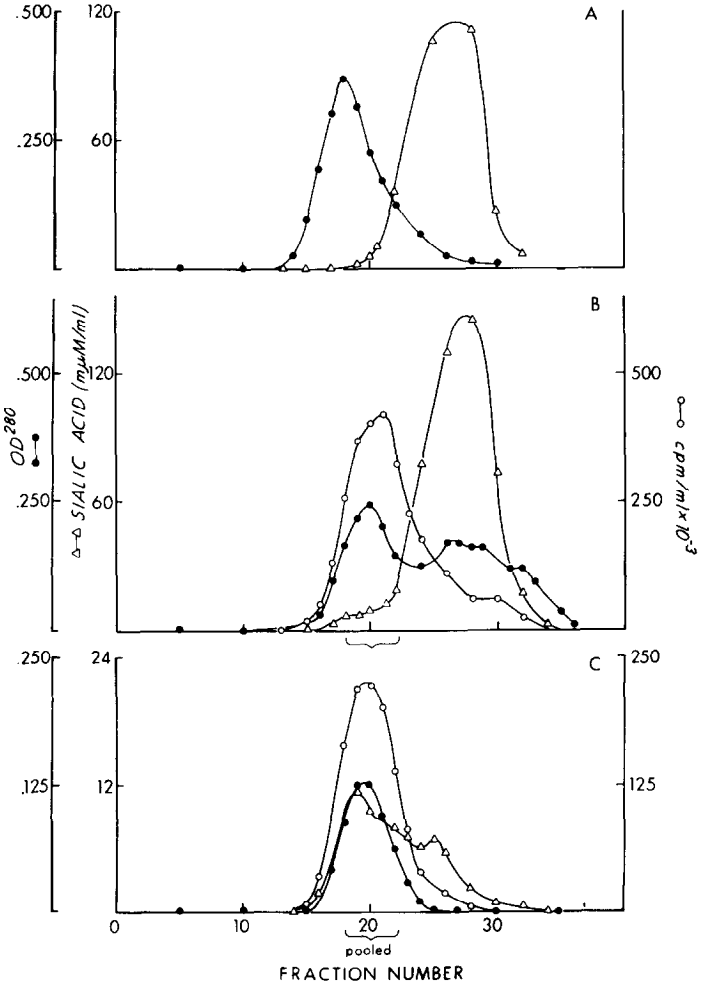

Fig. 2. Gel filtration of albumin on Sephadex G-150. A. Native albumin (0.5 μmole) was mixed with 2 μmoles of fetuin glycopeptide in the absence of coupling reagent and then applied to a Sephadex G-150 column (1.3x40 cm). The column was eluted with 0.9% NaCl and 2 ml fractions were collected. B. ^{131}I-albumin was coupled to fetuin glycopeptide as described in the "Methods" and then applied to the Sephadex G-150 column. C. The protein peak from (B) was pooled and reapplied to the same column. Δ——Δ, sialic acid; ●——●, O.D.$_{280}$; O——O, radioactivity.

lysozyme (Fig. 1). The glycopeptide-lysozyme was then further separated from uncoupled lysozyme and from the majority of the unreacted fetuin glycopeptide by chromatography on DEAE-cellulose. The coupled lysozyme, which retained 40% of its original enzyme activity, had a molar ratio of glycopeptide to protein of 1.5 to 1.

The coupled glycopeptide-albumin and unreacted albumin were separated from free glycopeptide by repeated gel filtration on Sephadex G-150 (Fig. 2B and 2C). The fact that free glycopeptide and albumin were completely separated in this system (Fig. 2A) established that nonspecific attachment of these two components did not occur. The material which was pooled after the second passage through the G-150 column contained about 30% fetuin glycopeptide-albumin and 70% free albumin. Over 90% of the sialic acid of the isolated fetuin glycopeptide-albumin was precipitable with 10% trichloroacetic acid while uncoupled glycopeptide did not precipitate under these conditions confirming the covalent nature of the linkage between the glycopeptide and albumin

Results:- The data in Table I summarize a series of experiments in which lysozyme, albumin, and their glycopeptide-coupled derivatives were injected intravenously into rats and the hepatic uptake of the compounds was determined after 10 minutes. Neither ^{131}I-labeled native lysozyme or albumin were removed by the liver (Expts. 1 and 7). Treatment of the native proteins with neuraminidase did not increase the hepatic uptake (Expts. 2 and 8). Similarly, the intact glycopeptide-proteins were not removed by the liver (Expts. 3 and 9). However, when the fetuin glycopeptide-proteins were treated with neuraminidase to remove the terminal sialic acid residues and then injected, the compounds were promptly removed by the liver (Expts. 4 and 10). Although the maximum uptake of asialofetuin-albumin was 28%, this represents uptake of 93% of the coupled molecules since only 30% of the albumin molecules contained a glycopeptide. Removal of the sialic acid residues of the fetuin glycopeptide results in the exposure of the penultimate galactose residues which, as Morell et al. (1) have shown, are the carbohydrate residues that are recognized by

TABLE I

HEPATIC UPTAKE OF COUPLED COMPOUNDS

Experiment	^{131}I Compound	% of injected radioactivity taken up by liver in 10 minutes
1	Native lysozyme	3.2
2	Native lysozyme treated with neuraminidase	3.6
3	Sialofetuin-lysozyme	3.5
4	Asialofetuin-lysozyme	34
5	Asialo-agalactofetuin-lysozyme	5.5
6	γG-lysozyme	1.2
7	Native albumin	1.0
8	Native albumin treated with neuraminidase	1.5
9	Sialofetuin-albumin	2.7 (9%)*
10	Asialofetuin-albumin	28 (93%)*
11	Asialo-agalactofetuin-albumin	11.4 (38%)*
12	γG-albumin	2.2

Male albino Wistar rats, 200-350 gm, were injected in either the tail vein or dorsal penile vein with 0.1 ml of 0.9% NaCl containing .01-.04 mg of ^{131}I-labeled glycopeptide-protein. After 10 minutes the injected rat was killed by cervical dislocation and its liver excised and irrigated through the hepatic vein with saline until the color of the liver changed to light tan. The liver was then weighed and counted in a Packard autogamma counter to determine radioactive uptake. All figures given are average results of at least two separate injections.

Incubations were carried out at 37°C under a toluene atmosphere as follows:

A. The compounds were incubated in 0.9% NaCl-0.02M phosphate-citrate buffer pH 6.2 containing 50 units/ml neuraminidase. The release of free sialic acid was determined by the thiobarbituric acid test (8). Incubations of 14 hours resulted in greater than 85% release of sialic acid.

B. Aliquots of the above reaction mixtures were brought to pH 4.5 with 0.5 M citric acid and citrate buffer pH 4.5 was added to a final concentration of 0.005 M. The reaction mixture was divided into 2 aliquots and 8 units of β-galactosidase were added to one aliquot. Incubation for 20 hours resulted in release of all the exposed galactose residues in the mixture containing enzyme. Free galactose was determined with galactose dehydrogenase.

*The figures in parentheses were calculated on the assumption that only coupled molecules were susceptible to uptake.

the hepatocyte receptors. When the galactose residues were removed from the asialofetuin-proteins with purified β-galactosidase and the compounds then injected into the rats, the hepatic uptake of the proteins was markedly decreased (Expts. 5 and 11). These findings provide strong evidence that it is the glycopeptide portion of the glycopeptide-protein complex which causes the hepatic uptake of the compounds.

In contrast to these results obtained with fetuin glycopeptide, the maxi-

mum uptake of γG glycopeptide-lysozyme and γG glycopeptide-albumin was 1.2% and 2.2%, respectively (Expts. 6 and 12). This result was not unexpected since we have recently shown that the glycopeptide obtained from human γG immunoglobulin contains an exposed galactose residue (3), and yet γG immunoglobulin circulates with a half life of 23 days (9). To determine if the γG glycopeptide-proteins "home" to organs other than the liver, we removed and counted numerous tissues of the injected rats but were unable to show selective organ uptake of these coupled proteins.

The specificity of the hepatocyte receptors for the asialofetuin-proteins was examined in another way by injecting into the rats various potentially inhibitory compounds just prior to the injection of the labeled compounds. The results of these experiments are summarized in Table II. Asialofetuin and

TABLE II

EFFECT OF VARIOUS COMPOUNDS ON THE HEPATIC UPTAKE OF ASIALOFETUIN-PROTEINS

Inhibitor	Amount	10-minute hepatic uptake of ^{131}I-asialofetuin-albumin (%)	10-minute hepatic uptake of ^{131}I-asialofetuin-lysozyme (%)
None		28	36
Heat denatured albumin	18 mg	--	35
Sialofetuin glycopeptide	0.42 μm	23.5	34
Asialofetuin glycopeptide	0.42 μm	9	12
Asialo-agalacto-fetuin glycopeptide	0.42 μm	--	31
Sialofetuin	5 mg	23	32
Asialofetuin	5 mg	2.1	7.5
γG-glycopeptide	0.96 μm	22	30
N-acetyllactosamine	0.43 μm	--	37

Asialo- and agalacto-inhibitors were prepared as described in Table I.

asialofetuin glycopeptide markedly decreased the hepatic uptake of both the labeled asialofetuin-proteins. Removal of the terminal galactose residues from the inhibitory compounds abolished their inhibitory activity. Intact fetuin and fetuin glycopeptide lacked significant inhibitory activity as did the γG glycopeptide and N-acetyllactosamine (galactose $\xrightarrow{\beta 1,4}$ N-acetylglucosamine) Since the γG glycopeptide contains at its nonreducing end the carbohydrate sequence galactose $\xrightarrow{\beta 1,6}$ N-acetylglucosamine $\xrightarrow{\beta 1,2}$ mannose (3), it is clear that more than an exposed galactose residue is required for a glycopeptide to be recognized by the hepatocyte receptor. The arrangement of the carbohydrates in the "core" of the glycopeptides (mainly mannose and N-acetylglucosamine residues) must also influence biologic activity. Heat denatured albumin, in an amount sufficient to significantly block phagocytic activity of the Kuppfer cells (10), had no inhibitory effect, suggesting that it is the liver parenchymal cells which are removing the glycopeptide-proteins from the circulation. This finding is consistent with the observations of Morell et al.(1).

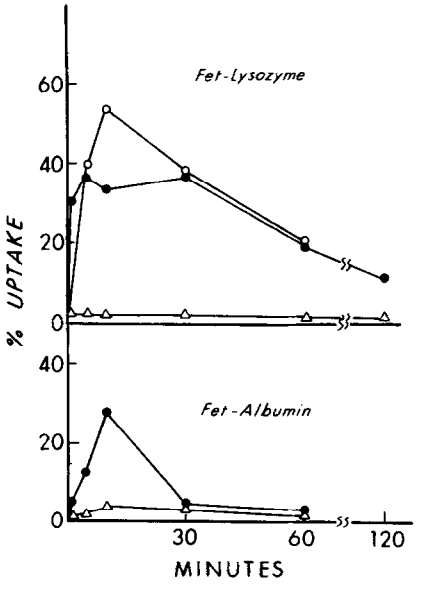

Fig. 3. Time course of the uptake and persistence of ^{131}I-labeled fetuin-proteins in the liver. The coupled compounds were injected at 0 time and the livers were removed at the time intervals shown and analyzed for radioactivity. Top. ^{131}I-fetuin-lysozyme: O——O, asialofetuin-lysozyme: ●——●, asialofetuin-guanidinated lysozyme; Δ——Δ, sialofetuin-lysozyme. Bottom. ^{131}I-fetuin-albumin; ●——●, asialofetuin albumin; Δ——Δ, sialofetuin-albumin.

The time course of hepatic uptake of the various fetuin-proteins is shown in Fig. 3. Each of the compounds was rapidly removed from the circulation with the maximal hepatic uptake occurring at about 10 min. The radioactivity then left the liver presumably indicating degradation of the proteins. Of note is the finding that the fetuin-lysozyme complex was degraded more slowly than the fetuin-albumin. The rate of degradation of fetuin-lysozyme and fetuin-guanidinated lysozyme was similar. When livers were removed 10 minutes after the injection of the protein and fractionated into their subcellular components, it was found that 90% of the radioactivity was in the particulate fractions, especially the lysosomal and microsomal fractions. Gregoriadis, et al. (11) have also found that asialoceruloplasmin is rapidly degraded in the hepatocytes.

Discussion:- The data presented in this paper demonstrate that the asialo-glycopeptide of fetuin covalently linked to either lysozyme or albumin causes rapid and selective removal of these proteins from the circulation by the liver, most likely by hepatocytes rather than Kuppfer cells. Thus the coupling technique offers a feasible method for directing the hepatic uptake of various proteins that otherwise would not "home" to the liver. In addition, it may also be possible to use this technique to specifically induce the hepatic uptake of other substances such as drugs.

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