Polyethylene glycol (PEG) is attached to proteins in order to increase their half-life in the circulation and reduce their immunogenicity in vivo. For many applications involving “targeting” molecules, it is important to know how PEG modification of the molecule affects its interaction with a receptor and the subsequent internalization, intracellular transport, and lysosomal degradation.

As a model system, we used asialofetuin, which binds to the galactose receptor of hepatocytes, because removal of sialic acid exposes galactose residues. We modified asialofetuin by attaching various amounts of PEG of molecular weight 1900 or 5000. The preparations were labeled with $^{125}$I so that endocytosis and degradation could be followed in suspended hepatocytes.

Depending on the number of PEG molecules attached, receptor-mediated uptake was affected to varying degrees. If two-thirds of the exposed amino groups of the asialofetuin molecule were modified, the rate of uptake decreased to less than one-fourth of controls; degradation of endocytosed molecules was 12% of controls. The reduction in endocytic uptake was due to a reduced rate of formation of the receptor-ligand complex.

Subcellular fractionation in density gradients showed that PEG-modified asialofetuin is transported intracellularly and degraded in the same manner as the native protein, but the rate of proteolysis is reduced. This observation explains the paradoxical result of experiments with injection of modified asialofetuin into rats in vivo: even though the clearance of one preparation of PEG-asialofetuin was much slower than that of the native protein, accumulation of radioactivity in the liver from the modified protein was twice as high. The hepatocytes accounted for 85% of the hepatic accumulation of either PEG-modified or native asialofetuin in vivo.

There is currently a great deal of interest in modifying proteins for in vivo use with polyethylene glycol (PEG) in order to reduce their immunogenicity and/or binding to pre-existing antibodies (Ashihara et al., 1978; Koide et al., 1982; Savoca et al., 1979; Suzuki et al., 1982; Anderson and Tomasi, 1988; Katre, 1990; Matsumiha et al., 1992). Another useful property of PEG-modified proteins is a prolonged half-life in the circulation (Liu et al., 1982; Knauf et al., 1988; Oda et al., 1989; White et al., 1989; Fujita et al., 1991; Meyers et al., 1991; Tanaka et al., 1991). PEG modification has also been used in order to make certain enzymes soluble in organic solvents (Takahashi et al., 1984a, 1984b).

The prolonged survival time in the circulation of PEG-modified proteins may be a result of reduced renal filtration and reduced binding to receptors; slower passage through the endothelial barrier of blood vessels might also be a factor.

Comparatively little work has been done to elucidate the influence of PEG modification on receptor binding; degradation of PEG-modified proteins has hardly been studied at all. In order to examine these aspects of PEG modification, we chose the asialoglycoprotein receptor system as a model, because it has been thoroughly studied (Schwartz, 1984), and PEG may be attached to asialofetuin without affecting the galactose residues which bind to the hepatic receptor. The mechanism of asialoglycoprotein uptake by hepatocytes is closely similar to other forms of receptor-mediated endocytosis, particularly those that show calcium-dependent receptor binding and subsequent delivery of the ligand to the lysosomes; an example is the LDL receptor system (Brown and Goldstein, 1986).

If a protein is to be used as a vehicle for targeted delivery of a radioisotope or a drug to diseased tissue, the rate of degradation of the protein vehicle may be an important determinant of the usefulness of the system. Slow proteolysis would be desirable if the transported moiety leaves the target cell on degradation of the vehicle (example: delivery of $^{111}$I to tumor tissue by means of iodine-labeled antibodies); on the other hand, if the active molecule has to leave the vacuolar system for the cytoplasm in order to be effective, then rapid degradation of the carrier protein is required (Hurwitz et al., 1980; Mosley et al., 1981; Furuno et al., 1982; Shen et al., 1985). As PEG modification of proteins may alter their intracellular transport and rate of lysosomal proteolysis, we wanted to study the kinetics of degradation of PEG-modified proteins.

Materials and Methods

Monomethoxypolyethylene glycol (MeO-PEG) of M, 1900 and 5000, 2,4,6-trichloro-s-triazine and fetuin were obtained from Sigma. Fetuin was desialylated by treatment with neuraminidase (Tolle- shaug, 1981).

Hepatocytes—Rat liver cells were prepared by Seglen’s (1976) modification of the method of Berry and Friend (1969). Hepatocytes were separated from nonparenchymal cells by centrifuging: They were incubated in a minimal salt medium containing 1% bovine serum albumin at a density close to 7 x 10⁶ cells/ml (Tolle- shaug et al., 1977).

Modification of Asialofetuin—PEG-modified asialofetuin was prepared by a modification of the method of Abuchowski et al. (1977). 0.95 g (0.5 mmol) of MeO-PEG of M, 1900 was dissolved in 10 ml of
dioxane. 92 mg (0.5 mmol) of 2,4,6-trichloro-s-triazine was added and then 1.25 mmol of NaOH as five portions of 50 μl of 6 M aqueous solution, with rapid stirring. After 30 min at room temperature, the reaction was stopped by the addition of 0.15 ml of glacial acetic acid. The mixture was then extracted with 2 ml of hexane-dichloromethane (1:1). The resulting solution was evaporated to dryness under nitrogen, and re-crystallized from methanol-water (1:1) with a constant boiling temperature of 80 °C. The product was separated from the母 matrix by filtration and purified on a thin-layer chromatography plate (Kieselgel 60 F254; Merck) with a solvent system of methanol-chloroform (1:1). The product was isolated as a white solid, mp 128-130 °C (dec), yield 90%. The purity of the product was determined by high-performance liquid chromatography (HPLC) on a C18 column (Phenomenex, USA) using a solvent system of methanol-water (90:10) at a flow rate of 1 ml/min and a detection wavelength of 254 nm. The purity was found to be 98%.

Modification of PEG-5000 was carried out by exactly the same procedure, using the same number of moles of PEG.

Asialofetuin was incubated with 0.1 ml of 0.1 M suc cinic acid anhydride in dioxane with 0.6 ml of asialofetuin solution (5 mg/ml) in 0.2 M sodium carbonate buffer, pH 9.5. The solution was left overnight and dialyzed against phosphate-buffered saline.

Labeling with 125I—Asialofetuin was labeled with 125I by a modification (Tolleshaug, 1986) of the sodium hypochlorite oxidation method of Redshaw and Lynch (1974).

For the introduction of 125I-tyramine-cellobiose (125I-TC) into PEG-modified ligands, the following reaction was carried out: 10 mg of sodium borohydride was added to 1 ml of the remaining amino groups: 10 mg of sodium borohydride was added to 1 ml of a 0.3 mg/ml solution of PEG-asialofetuin in 0.2 M sodium carbonate buffer, pH 8.5. The solution was left in the refrigerator overnight, then filtered through a piece of laboratory film with a small hole in it, in order to retard the entry of oxygen. The tube was placed on a water bath at 60 °C, and 30 μl of 1 M octyl glucoside in water and 0.125 ml of 0.2 M sodium carbonate buffer, pH 9.5, were added. The tyramine-cellobiose was iodinated and activated with trichlorotriazine, "activated-PEG" was used without further purification. Modification with PEG-5000 was carried out by exactly the same procedure, using the same number of moles of PEG.

Native asialofetuin labeled with the residualizing label 125I-TC was prepared by the method described by Hysing and Tolleshaug (1986). Unbound radioiodine was removed by gel filtration (PD-10 column from Pharmacia, Sweden) in phosphate-buffered saline containing 1 mM EDTA and 0.5 mg/ml human serum albumin, and the preparation was dialyzed extensively against the same buffer in order to remove the detergent.

Measurement of Uptake and Degradation in Hepatocytes—Degradation (acid-soluble radioactivity) was followed by mixing the 250-310 μg protein of the cell suspension with an equal volume of 10% trichloroacetic acid, followed by centrifuging. Uptake of labeled asialofetuin by the cells was determined by placing a 250-μl aliquot on top of 500-μl portions of the cell suspension with an equal volume of 10% trichloroacetic acid, followed by centrifuging. The cells were separated from the medium. The amount of radioactivity in the cell pellet is referred to as cell-associated radioactivity (Kolset et al., 1979). The uptake of asialofetuin by the cells, the amounts of cell-associated and acid-soluble radioactivity at a given time point were added, and the sum was called total uptake (degradation in the medium is negligible).

Cell Fractionation—Isopycnic centrifuging in a Nycodenz density gradient has been described in detail previously (Kindberg et al., 1984). Briefly, the washed hepatocytes were homogenized in a Dounce homogenizer, the nuclei were removed by differential centrifuging, and a portion of the resulting "cytoplasmic extract" was layered on top of Nycodenz (NY-O-MED, Oslo 4, Norway) gradients, which were spun at 25,000 rpm in a Kontron SW-28 rotor for 45 min at 4 °C. The lysosomal marker enzyme β-N-acetylglucosaminidase was measured as described by Barrett (1972).

Studies in Vivo—Protein concentrations were calculated from the absorbance at 280 nm. The dose was injected into the vena femoralis. 50-μl blood samples were taken into heparinized tubes at 5 min intervals. For determinations of the total radioactivity that had become associated with the liver, the organ was rapidly removed 40 min after the dose had been injected, and radioactivity/g liver was determined.

For determination of the radioactivity that was associated with three major types of liver cells, a trace dose (containing 14 x 106 cpm) of either 125I-labeled native asialofetuin or 125I-labeled PEG-1900-modified asialofetuin was injected. 20 min later, perfusion of the liver was initiated, and the liver cells were separated by centrifugal elutriation as described by Nenneset et al. (1988). The number of cells in each fraction was determined, and aliquots were removed for determination of radioactivity. The number of cells of each major type in the liver was taken from Blomhoff et al. (1984).

RESULTS

Preparation of PEG-modified Asialofetuin—Asialofetuin was reacted with activated PEG of M, 1900 or 5000 Da; the reaction mixture contained molar amounts equal to 0.3, 0.6, 1.0, and 2.0 equivalents of the available amino groups (lysine residues) in the asialofetuin molecule. Table I shows that modification with activated PEG-1900 resulted in 11, 37, 45, and 65% loss, respectively, in primary amino groups, which corresponds to the attachment of approximately 2, 6, 7, and 10 PEG strands/asialofetuin molecule. With activated PEG-5000, the highest molar ratio in the reaction mixture did not produce a higher degree of substitution than the next lower ratio (Table I). Steric hindrance may prevent a higher degree of substitution than about 60%.

Effects of PEG Modification on the Uptake of Asialofetuin in Hepatocytes in Vitro—When PEG is coupled to amino groups with triazine, the modified amino groups lose their basic properties. Whether this alteration in charge would influence on the uptake of asialofetuin in hepatocytes, was determined by incubating hepatocytes in the presence of 125I-labeled preparations of PEG-1900-asialofetuin (50% modified), MeO-ethanol-asialofetuin (60% modified), succinyl-asialofetuin (60% modified), or native asialofetuin as control. MeO-ethanol-asialofetuin is analogous to PEG-asialofetuin, except that the molecular weight of the side chains is only 77. In succinyl-asialofetuin, a negative charge is introduced on each modified amino group in the molecule.

Fig. 1 shows the total uptake of each ligand. Succinyl-asialofetuin was endocytosed by the cells with the same kinetics as the unmodified protein. After 20 min of incubation, PEG-asialofetuin was endocytosed in amounts corresponding to 54% of the control, while methoxyethyl-asialofetuin was taken up at a slightly higher rate than the unmodified protein. These results indicate that the charge in the charge of the ligand has a negligible influence on the uptake in hepatocytes, the charge of succinyl-asialofetuin being much more negative than that of native asialofetuin, while the uptake is the same. On the other hand, the charge difference between PEG-asialofetuin and MeO-ethanol-asialofetuin is negligible, but only the PEG modification of the ligand gave a much smaller uptake. This decrease must be due to the effects of the PEG strands.

Table I

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<tr>
<th>Molar ratio of asialofetuin after reaction with activated polyethylene glycol</th>
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a Molar ratio of activated PEG to amino groups in asialofetuin in the reaction mixture.

b As determined by the trinitrobenzen sulfonic acid reaction.
Endocytosis of PEG-modified Asialofetuin

Degradation of PEG-modified Asialofetuin by Hepatocytes in Vitro—Fig. 2B shows the time course of degradation of different preparations of PEG-1900-modified asialofetuin. After 45 min, PEG-asialofetuin (65% of the amino groups modified) is degraded in amounts of 52% of the control. As the degree of PEG-modification decreased to 45, 37, or 11%, preparations were degraded in amounts of 60, 72, and 78%, respectively, of the control. This means that the degradation of asialofetuin by isolated hepatocytes is progressively inhibited as the protein molecule is substituted with more PEG residues. This effect is independent of the inhibition of endocytosis which is observed with the same ligand preparations. The same results were obtained with asialofetuin modified with PEG of M, 5000 (Fig. 3).

In order to determine the effect of PEG modification on degradation by a method that is completely independent of any effect on the uptake, suspensions of hepatocytes were incubated with the different 125I-labeled PEG-asialofetuin preparations and native asialofetuin as control (Tolleshaug et al., 1980). After 20 min, the suspensions were chilled and the cells were washed in an ice-cold medium containing 5 mM EGTA in order to remove the surface-bound ligand. The suspensions were reincubated at 37 °C and degradation followed. The washing step served to stop the uptake of asialofetuin, which might distort the degradation measurements. In experiments of this design, PEG-1900-asialofetuin (56% modified) was degraded at 60% of the control rate; a preparation that was 19% modified was proteolyzed at 78% of the rate of native asialofetuin (Fig. 4) (see also Fig. 5, which shows experiments done by the same procedure).

Inhibition of Intracellular Transport of PEG-modified Asialofetuin—In order to determine the intracellular transport of the PEG-modified protein, two types of experiments were performed: first, the influence of the proteolytic inhibitors 1900-modified asialofetuin preparations and native asialofetuin as control. These results indicate that the uptake of the ligand is decreased, depending on the number of PEG molecules attached. After 40 min, PEG-asialofetuin (65% modified) was endocytosed by the cells to the extent of 24% of the control. As the number of attached PEG molecules decreased to 7, 6, or 2 (Table I), the PEG-modified preparations were endocytosed in amounts of 54, 68, and 79% of the control, respectively. These results indicate that the uptake of asialofetuin by the galactose-specific receptor of hepatocytes is progressively inhibited as more PEG residues are attached to the protein, even though the galactose moieties are not modified. Closely analogous results were obtained with asialofetuin modified with PEG of M, 5000 (Fig. 3).

Degradation of PEG-modified Asialofetuin by Hepatocytes

![Fig. 1. Uptake of native asialofetuin, succinyl-asialofetuin, methoxy-ethyl-asialofetuin, and PEG-modified asialofetuin in suspended hepatocytes.](image1)

![Fig. 2. Effect of modification with PEG-1900 on the uptake and degradation of asialofetuin in suspended hepatocytes.](image2)

![Fig. 3. Effect of modification with PEG-5000 on the uptake and degradation of asialofetuin in suspended hepatocytes.](image3)
chloroquine and leupeptin was investigated, and second, subcellular fractionations were performed.

The bacterial tripeptide derivative, leupeptin, and the weak base, chloroquine, are proteolytic inhibitors with different effects on the degradation of asialofetuin (Berg and Tolleshaug, 1980; Tolleshaug and Berg, 1981). Fig. 5 shows the results in which the cells were reincubated with 0.05 mg/ml chloroquine and 0.2 mM leupeptin. Fig. 5A shows the degradation of native asialofetuin, while Fig. 5B shows the degradation of PEG-asialofetuin (45% modified). The results indicate that both chloroquine and leupeptin inhibit the degradation of native as well as PEG-modified asialofetuin by about 50%. These results provided an indication that PEG-modified asialofetuin is transported intracellularly and degraded in the same manner as the native protein, even though the rates are reduced.

Intracellular Distribution of PEG-modified Asialofetuin—In order to obtain the complete picture of intracellular distributions of asialofetuin, the labeled degradation products should be trapped in the intracellular organelles where they are produced. In the present study, we used 125I-tyramine-cellobiose (125I-TC) (Pittman et al., 1983; Tolleshaug and Berg, 1981; Hysing and Tolleshaug, 1986). Different suspensions of hepatocytes were incubated with native or PEG-modified asialofetuin which were labeled with 125I-TC. The initial incubation was for 20 min at 37 °C, followed by a wash in buffer containing 5 mM EGTA. Aliquots of the cell suspension were removed after reincubation for 5 or 90 min. The cells were washed, homogenized, and the post-nuclear supernatant was fractionated by centrifuging in an isotonic density gradient.

Fig. 6 shows the distributions of acid-soluble and acid-precipitable radioactivity from the two ligands. Fig. 6A shows the distribution curves for PEG-asialofetuin (45% modified) and native asialofetuin after 5-min reincubation. In these gradients, nearly all radioactivity is acid-precipitable. The peak activity of the lysosomal marker enzyme β-N-acetylgalcosaminidase was at 1.13 g/ml (data not shown). The distribution curves indicate early endosomes (1.05 g/ml), multivesicular bodies (1.09 g/ml), and lysosomes (1.13 g/ml) (Kind-
H., Iodine-labeled native or PEG-1900-modified asialofetuin (50% modified) was removed for determination of cell-associated radioactivity. Up to about 40 min, the amount of surface-bound radioactivity increased in a linear fashion (Fig. 7) (within experimental uncertainty). The rate of binding of PEG-1900-modified asialofetuin was only 15% of the rate of native protein.

**Binding of PEG-modified Asialofetuin to Hepatocytes at Low Temperature**—Aliquots of the cell suspension was chilled to 0 °C. At this temperature, internalization is so slow as to be undetectable. Iodine-labeled native or PEG-1900-modified asialofetuin was added, and samples of the suspensions were removed for determination of cell-associated radioactivity. Up to about 40 min, the amount of surface-bound radioactivity increased in a linear fashion (Fig. 7) (within experimental uncertainty). The rate of binding of PEG-1900-modified asialofetuin was only 15% of the rate of native protein.

**Clearance of PEG-modified and Native Asialofetuin in Vivo**—Ten million cpm of 125I-labeled proteins was injected into rats weighing about 400 g. The amount of protein was 100 µg; this dose of asialofetuin has a half-life of close to 3 min (Charlwood et al., 1979). Blood samples were taken; after 40 min, the animals were killed and the amount of radioactivity in the liver was determined. Fig. 8 shows the total amount of radioactivity in the blood. The slow rise after about 10 min is caused by the release of degradation products (iodide ions) from the liver (the “trapped label” was not used).

The results shown in Fig. 8 indicate that the control preparation (unmodified asialofetuin) has a half-life in the blood of about 3 min, while PEG-asialofetuin (43% modified) has a half-life of about 7 min. In the liver, the amount of radioactivity from PEG-modified protein was twice as high as from native asialofetuin (Fig. 9) after 40 min. Slower degradation (Fig. 3) as well as a considerable delay in the uptake (Fig. 8) contributed to the increase in hepatic recovery of modified asialofetuin at the end of the experiment.

**Distribution of PEG-modified and Native Asialofetuin between the Main Types of Liver Cells**—Fourteen million cpm of 125I-labeled proteins was injected into rats. Twenty min after the injection, parenchymal, endothelial, and Kupffer cells were isolated from the liver at room temperature. Total recoveries of radioactivity in the liver from either protein preparation was substantially as shown in Fig. 9. The distribution of radioactivity between the various types of liver cells may be somewhat distorted by degradation, including continuing degradation at the temperature of the elutriation procedure; for instance, the degradation of ovalbumin (a glycoprotein) in vivo was much faster in parenchymal cells than in endothelial cells (Kindberg et al., 1990) within 20 min of the injection.

In the parenchymal cells, the proportion of radioactivity from both protein preparations was slightly less than 90% (Fig. 10). The percentage of PEG-modified protein in the endothelial cells was more than twice as high as the percentage of native protein; on the other hand, the uptake of native protein in the Kupffer cells was twice as high as the uptake of PEG-modified asialofetuin.

**DISCUSSION**

In certain important receptor systems, reactive lysine residues of the ligand forms an important part of the receptor-binding portion of the molecule; examples are low density lipoprotein (Brown and Goldstein, 1986) and insulin (Knutson, 1987). In these cases, modification of amino groups leads
to a decrease in affinity for the receptor. In the present system, attaching PEG residues to some of the amino groups of the ligand does not modify the galactose residues which bind to the receptor. Accordingly, any effect on the rate of uptake must be due to steric hindrance by the bulky PEG molecules.

It has been shown that the rate-limiting step in the uptake of asialoglycoproteins by hepatocytes is binding of the ligand to its receptor (Tolleshaug, 1981). The rate of internalization of the receptor-ligand complex would not be expected to change due to the presence of PEG on the ligand. Thus, a reduction in the uptake following attachment of PEG would be presumably due to a decreased rate of formation of the ligand-receptor complex. This was confirmed by measuring the rate of binding at low temperature, which was reduced to one-seventh of the rate of the native protein. The reduction was, in fact, much greater than expected from the reduction in the rate of uptake following attachment of PEG to its receptor (Tolleshaug, 1981).

Modifying asialofetuin by as little as three PEG-1900 molecules causes a measurable decrease in the rate of uptake; about 8 of these residues leads to a decrease of 50%. If a reduction of this magnitude is caused solely by a reduction in the free energy of binding, the reduction is less than 2 kJ/mol, which is less than the free energy involved in the formation of a single hydrogen bond. Thus, the observed magnitude of the changes in the rate of uptake on PEG modification are compatible with steric hindrance by PEG residues (Tolleshaug et al., 1980).

A reduction in the rate of degradation would also be expected. By introducing triazine groups (the coupling agent for TC) on the lysine residues, we removed potential cleavage points for the important lysosomal protease cathepsin B. As a result of steric hindrance, the peptide chain would also be less accessible to proteases in general. An effect on intracellular transport would also be a possibility, as PEG residues might reduce the adherence of the ligand to membrane proteins, including receptors, of the vacuolar system. A lower degree of adherence might also lead to the release of a larger fraction of intact ligand molecules from the cells through diacytosis (retro-endocytosis) (Tolleshaug et al., 1981; Tolleshaug, 1984).

Modification of about 12 out of 16 amino groups reduced the rate of degradation of intracellular ligand by 58% in isolated cells. No effect on intracellular transport could be determined, either by using specific inhibitors or by examining intracellular distributions at different time points. As regards the retro-endocytic pathway, PEG modification produced a measurable increase in the fraction of released intact ligand molecules (not shown), but the change was far too small to explain the observed decrease in the rate of degradation. We are left with the inhibition of the action of cathepsin B or other proteases as the most likely explanation of the reduced degradation.

Following separation of the main cell types of the liver by centrifugal elutriation, more than 85% of the hepatic recovery of either native or PEG-modified asialofetuin was localized to the hepatocytes. A slightly higher proportion of injected asialofetuin molecules was found in the hepatocytes in a quantitative electron microscopic study (Hubbard et al., 1979). In endothelial or Kupffer cells, the significance of the variations between the recoveries of label from PEG-modified versus native asialofetuin is not clear, as the recoveries in these cell types are still fairly small.

The effect of PEG-modification on the rate of clearance of asialofetuin was much smaller than on the rate of clearance of other PEG-substituted proteins of therapeutic interest. Some of these proteins are taken up by the liver by poorly characterized systems (Smit et al., 1987). Alterations of the charge of the protein certainly influences endocytosis; as with PEG-asialofetuin, steric interference with binding to cells is very important (Mori et al., 1991).

In experiments in vivo, the PEG-modified protein left a significantly increased amount of radioactivity in the liver, compared to the native protein, even though modification also reduced the rate of uptake. The degree of increase in the retention of radioactivity could not be predicted on the basis of rates of uptake and degradation as determined in cells in suspension, as these rates decreased in approximately equal measure in isolated cells. The reduced degradation would be beneficial in many potential applications of PEG-modified proteins, e.g. delivery of lysosomal enzymes. If the aim was to release small molecules from a protein carrier (Hurwitz et al., 1980; Moseley et al., 1981; Furuno et al., 1982; Shen et al., 1985) subsequent to degradation, then slow proteolysis would be deleterious.

In targeting radiolabeled ligands for therapeutic or diagnostic purposes (e.g. scintigraphic imaging), decreased degradation might, on one hand, increase the amount of label in the target cells (Morimoto and Fujimoto, 1985); on the other hand, retention of more nonspecifically accumulated label in the liver or spleen would be a definite drawback (Goldenberg, 1987; Meares et al., 1988).

REFERENCES
Endocytosis of PEG-modified Asialofetuin