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UNIVERSITY OF ALBERTA

The Carbohydrate Modification and Radiolabeling of Monoclonal Antibodies

by

Pei Qi

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF **Doctor of Philosophy**

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TO MY PARENTS FOR THEIR CONTINUOUS ENCOURAGEMENT AND SUPPORT TO MAKE THIS HAPPEN

Abstract

In an effort to better understand the biological effect of the carbohydrate portion of antibodies, N-Acetylgalactosamine (GalNAc), Gal-GalNAc and sialic acid have been conjugated to human IgG (hIgG), which served as a model antibody for these studies. Novel hexafluoro side-chain derivatives of GalNAc and Gal-GalNAc were prepared and used for the conjugation onto hIgG. The number of sugar residues on the antibody molecule was simply determined by integration of calibrated ^{19}F-NMR signals from the analysis of the purified conjugates. For sialic acid, a linking derivative containing an aliphatic aldehyde functionality was synthesized and the final conjugate was analyzed by a resorcinol assay. Biodistribution studies were conducted in normal male ICR mice using radioiodinated sugar conjugates, and unmodified hIgG as the control. Enhanced liver uptake was observed at the early time periods after injection with an associated change in circulating blood level for the Galactose-based hIgG preparations suggesting preferential uptake by the hepatic carbohydrate receptors. However, no significant differences were observed in the biodistribution for the sialic acid conjugates.

Furthermore, the carbohydrate moiety of two antibodies, hIgG and MAb 155H.7 (IgG_{2b}) were modified with periodate treatment followed by condensation with amino and hydrazine compounds. The tissue distribution data of modified antibodies by the above treatment in normal mice showed that there were no apparent differences observed in blood level and liver uptake after intravenous injection at least at an early stage. The data from these studies indicated that the carbohydrate portion of the IgG molecule may not play an important role for its catabolism and clearance in the mice.

A novel chelating agent, para-aminobenzylpropylenediaminetetraacetic acid (PAB-PDTA) was conjugated to hIgG and MAb 155H.7 *via* the carbohydrate moiety of the antibodies. The conjugation was achieved by reaction of PAB-PDTA with the aldehyde groups generated from the periodate oxidation of the antibody molecule. The PDTA-MAb 155H.7 conjugate was evaluated for it's antigen binding

ability by ELISA assay. There were no apparent changes observed in immunoreactivity by this modification. Biodistribution of ^{111}In-labeled PDTA-antibody conjugates showed a comparable tissue uptake to that of other published data with this antibody.

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List of Abbreviations

BSA	Bovine serum albumin
C	Constant region on IgG molecule
CDR	Complementarity-determining regions
CEA	Carcinoembryonic antigen
CMP	Cytidine monophosphate
d	Day
DD-H_2O	Double distilled, deionized water
DMF	Dimethyl formamide
DMSO	Dimethylsulfoxide
DNPH	2,4-dinitrophenylhydrazine
DTPA	diethylenetriaminepentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunoadsorbent assays
ERC	Edmonton Radiopharmaceutical Centre
EtOAc	Ethyl acetate
EtOH	Ethanol
FSA	Formamidine sulfinic acid
Gal	D-Galactose
GalGalNAc	Galß1,3GalNAc
GalNAc	N-Acetyl-D-galactosamine
Glc	D-Glucose
GlcNAc	N-Acetyl-D-glucosamine
h	Hour
HAMA	Human antimouse antibodies
HFGDE	Hexafluoroglutaric acid dimethyl ester
hIgG	Human immunoglobulin G
HPLC	High performance liquid chromatography
HSA	Human serum albumin
HSA-Tα	Galß1,3GalNAc-HSA

HV	Hypervariable region on IgG molecule
Ig	Immunoglobulin
Iodogen	1,3,4,6-tetrachloro-3α,6α-diphenylglycouril
ip	Intraperitoneal
ITLC	Instant thin layer chromatography
iv	Intravenous
M	Molar concentration
min	Minute
MAb	Monoclonal antibody
Me_2S	Dimethyl sulfide
MeOH	Methanol
NANA	N-Acetylneuramic acid, Sialic acid
NaOAc	Sodium acetate
NMR	Nuclear meganetic resonance
PDTA	Propylenediaminetetraacetic acid
PNB-PDTA	para-nitrobenzyl-PDTA
PAB-PDTA	para-aminobenzyl-PDTA
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PI	Post injection
RES	Reticuloendothelial system
SCA	Single-chain antigen-binding protein
SE HPLC	Size exclusion HPLC
SPECT	Single photon emission computed tomography
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
V	Variable region on IgG molecule
ϵ	Molar extinction coefficient (liter $mole^{-1}$ cm^{-1})
NH_4OAc	Ammonium acetate

1. INTRODUCTION

Since the introduction of the hybridoma technique in antibody production, numerous reports on laboratory studies and human clinical trials using monoclonal antibodies in immunoimaging and immunotherapy have been published. However, several problems still exist and prevent effective clinical application of radioimmunoimaging and therapy. High non-specific uptake, for example in liver and kidney, and actually very small amount of uptake of radiolabeled MAb in tumors are among the problems.

Galactose-conjugated antibodies were examined for the application in treatment of ovarian carcinoma or other malignant cells confined to the peritoneal cavity (Mattes et al., 1987). These investigators demonstrated that after injection there was rapid clearance from the circulation which supported the concept that selective modification of antibodies with specific carbohydrate structures can be an effective mechanism for the alteration of their biological behaviour and may lead to more effective targeting of these complexes to specific locations *in vivo*.

Additional problems in immunoscintigraphy relate to the high background radioactivities in non-target tissues such as liver, spleen, kidney and bone and the low uptake by tumors. In addition to improving the specificity of MAbs to target tumors, optimizing the ways antibodies are radiolabeled remains a most important objective. There are several reports indicating that antibodies are affected differently by various labeling procedures (Otsuka, et al., 1987) and different approaches may be needed for the labeling of individual antibodies (Eckelman, et al., 1986).

Because the carbohydrate moieties of antibodies are located almost exclusively on the constant (CH2) Fc region which is not related to the antigen-antibody binding process, it has been chosen by a number of investigators for the conjugation of chelating agents and other drugs. The conjugation of these reagents was carried out by conventional reductive amination including a reduction of the resultant Schiff base by $NaCNBH_3$. This important technology has been the subject of intensive examination by a number of industrial concerns.

In light of these considerations, the objectives of this work can be stated as follows:

1. To study the effect of additional sugar conjugation to antibodies on their *in vivo* and *in vitro* behavior.
2. To study the effect of structural modifications to the native carbohydrate moieties of antibodies on the biodistribution of the radiolabeled analogues.
3. To explore the usefulness of native antibody carbohydrates in the conjugation of a novel chelating agent.

2. SURVEY OF THE LITERATURE

2.1 Early Work in Tumor Localization of Radiolabeled Antibodies

Before the introduction of hybridoma technology, purified polyclonal antibodies were used in radioimaging studies to localize tumors. The idea of Pressman, et al. (1953) that antisera prepared against tumor tissues might contain antibodies capable of localizing in these tumors was generated from the previous reports which stated that antisera prepared against normal tissues of rats or mice localized in various organs of these animals *in vivo*. Pressman's results did show that there was a greater localization of radioactivity in tumors of the mice injected with radioiodinated globulin of anti-tumor sera than that of mice injected with normal serum preparation. However, the difficulty noted a higher background in normal tissues due to the nature of polyclonal antibodies posed the main problem. Consequently, efforts were made to use purified antibodies in order to improve localization in tumors. Such methods included treatment of radioiodinated globulin fractions with lymphosarcoma and subsequent elution of the adsorbed antibodies from the tissue (Korngold, et al., 1954; Pressman, et al., 1953); prior passage of antisera through a normal rat (Kyogoku, et al., 1964); and DEAE-A50-Sephadex separation (Day, et al., 1965). Later development of anti-carcinoembryonic antigen (anti-CEA) antibody entailed the affinity purification of antibodies using immunobilized CEA (Goldenberg, et al., 1974; Hoffer, et al., 1974; Belitsky, et al., 1978; Dykes, et al., 1980). However, the difficulties in obtaining reasonably pure polyclonal antibodies and the lack of specifity to tumor targets prevented further development of these techniques.

The early work in radiolabeling of antibodies was almost exclusively done with radioiodine, ^{131}I for radioimaging and ^{125}I for autoradiography. Since tumors have the property of accumulating a wide variety of foreign substances non-specifically, ascertaining the localization of antibodies in tumors presented a big challenge. It was reported that this non-specific background was sometimes so great that it had been utilized diagnostically for locating tumor metastases (Pressman, et

al., 1957). Paired labeling techniques in which ^{131}I ($T_{1/2}$ = 8.1 days) and ^{133}I ($T_{1/2}$ = 22 hours) were used to label an anti-tumor antibody and a normal antiserum control. These were injected simultaneously thus permitting the direct measurement of tumor-localizing antibodies over and above non-specific background localization of control material. In other words, the experimental subject served as its own control (Pressman, et al., 1957). This was described as a localization index.

2.2 Hybridoma Technology and Monoclonal Antibody (MAb) Production

2.2.1 Antibody Structure and the Conventional Approach to Antibody Production

A model structure of a typical IgG molecule is presented in Fig. 2.1 (modified from Zuckier, et al., 1989). It is composed of two identical heavy (H) and two identical light (L) polypeptide chains that are covalently linked by disulfide bonds. The side chain groups of amino acid residues in the heavy and light chain variable (V) regions fold and interact with each other to assemble the complementary determining and antigen-binding sites. Antibodies that react with different antigens differ from each other in the sequence of their V regions,

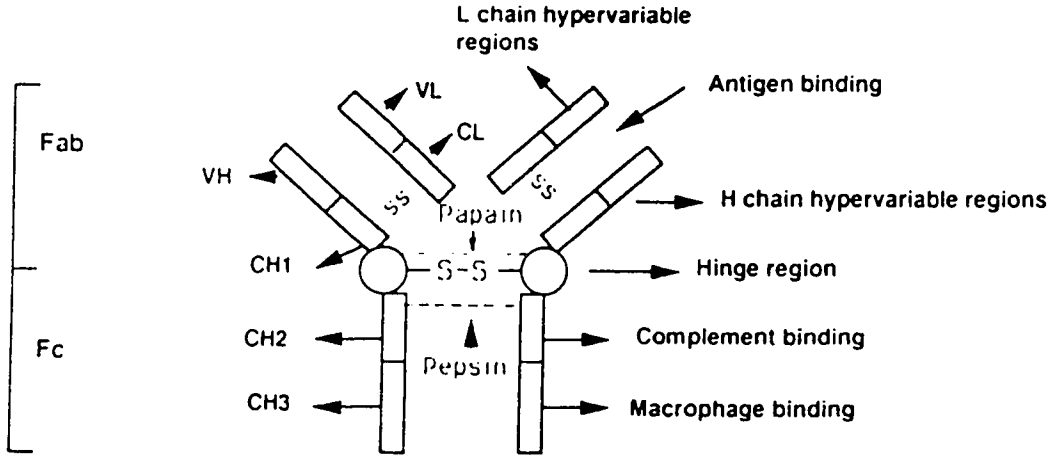


Fig. 2.1 A model of a typical IgG molecule. Papain and Pepsin cleavage sites are indicated by dotted line

especially in hypervariable (HV) regions. Different antibody classes (IgM, IgD, IgG, IgE, IgA) and their subclasses possess different constant (C) regions while the amino acid sequence of the C region is highly conserved among antibodies within each class and subclass.

The conventional method of producing an antibody is by injection of an antigen into an experimental animal and the subsequent collection of its serum. Higher organisms have evolved an immune response in order to protect themselves from a seemingly infinite array of infectious and potentially toxic agents in the environment. A normal individual can probably produce over a billion different antibodies, each of which has different amino acid sequence in its V and hypervariable regions (Zuckier, et al., 1989). Each antibody-producing cell makes one species of antibody. The serum contains a mixture of antibodies each produced by different clones of B lymphocytes since most antigens have various antigenic determinants. Therefore, using the conventional immunization method, it is very difficult to generate large amounts of homogenous antibody. It is virtually impossible to repeatedly replicate an exact antibody response.

2.2.2 Hybridoma Technology

Because each antibody is produced by a single plasma cell line, if the cell line can be separated and cultured MAbs of exactly the same class and reactivity would be produced. Pioneer work led by Kohler and Milstein (1975) demonstrated the manufacture of predefined specific antibodies by means of permanent tissue culture, namely the hybridoma technique. The successful fusing of a B lymphocyte with a mouse myeloma cell produced a hybrid clone which can continue to grow in culture solved the problem of B lymphocyte mortality.

The general procedure in the production of MAb is as follows: Animals (usually mice or rats) are immunized with the desired antigen. The spleen or lymph cells of the animals showing an immune response are then separated. These cells are fused with a myeloma cell line by the addition of polyethylene glycol (PEG) which promotes membrane fusion. Only a small proportion of the cells may actually

be fused successfully. The fusion mixture is then incubated in a medium containing 'HAT' which is a mixture of hypoxanthine, aminopterin and thymidine. Aminopterin is a powerful toxin which blocks a metabolic pathway, causing myeloma cell death. However spleen cells can bypass this by providing the intermediate metabolites, hypoxanthine and thymidine, and eventually die naturally after 1-2 weeks. Only fused cells survive as they have the immortality of the myeloma and the metabolic bypass of the spleen cells. The fused cells are then screened for their ability to produce antibodies. The antibody producing cells are plated out so that only one cell is cultured in each well. Because MAb is secreted from clones of identical hybridoma cells it is homogenous. The selected specific hybridoma clone can be grown in culture to produce MAb in the supernatant or they can be introduced into mice to grow whereby up to 5-20 mg/mL of MAb can be produced in the resultant ascites fluid.

Numerous reports on laboratory tests and human clinical trials using MAbs in immunoimaging and immunotherapy have been published. In addition to the introduction of radioactive atoms, cytotoxic drugs, and enzymes, many other chemicals have been attached to MAbs for use in a variety of fields. Thus, the development of monoclonal antibody technology has provided a bright prospect in development of immunodetection and immunotherapy.

2.2.3 Problems Associated with Use of Monoclonal Antibodies

A common problem encountered in MAb use is the murine antibody identity for most available MAbs. When individuals are challenged by a foreign protein, such as a murine MAb, the human antimouse response will occur in the majority of patients (Divgi, et al., 1989; Reynolds, et al., 1989). The existence of human antimouse antibodies (HAMA) may alter the kinetic distribution of subsequently administrated MAb (Pimm, et al., 1990; Stewart, et al., 1990).

The HAMA response has much lower frequency in patients receiving MAb from their own B-cell lymphomas (Divgi, et al., 1989). Human antibodies offer several advantages over their murine counterparts (Ho, et al., 1987). First, human

antibodies react with specific antigen *in vivo* more effectively because of reduced likelyhood of cross-reaction with normal human tissue than mouse antibodies. Second, human antibodies appear to be less antigenic in humans than mouse antibodies. Since the development of the first human MAb (Steinitz, et al., 1977) a number of human MAbs have been produced. However, several problems exist. Human cell lines from tumor-bearing patients used as fusion partners for human-human hybridomas are not as stable or as productive as murine myeloma cell lines. It is not generally possible to hyperimmunize humans before harvesting the B-cells for fusion, therefore, most human monoclonal antibodies are of the IgM isotype which generally have a weak affinity for the target antigen (Sahagan, et al., 1986). In addition, in comparison to the IgG isotope, IgM antibodies (from human or murine sources) have reduced tissue perfusion characteristics due to these higher molecular weight and are metabolised much faster. The difficulties encountered in making human monoclonal antibodies have induced scientists to seek alternatives.

2.2.4 Recent Developments in Antibody Production

It has been known that antibodies are composed of V and constant (C) domains in which the V domains define the antigen specificity, while the C domains contain the antigenic determinants. An alternative is to produce antibodies capable of escaping surveillance by the human immune system, while retaining the tumor specifity of a murine MAb. This is accomplished by the production of genetically engineered murine/human chimeric MAbs. By this technique, a humanized MAb is produced by fusing V region exons of a mouse MAb to the C region of an human antibody through recombinant DNA technology. Stable transfected murine myeloma cell lines are then used to produce intact chimeric mouse/human antibodies (Oi, et al., 1986; Shaw, et al., 1988; Sahagan, et al., 1986). *In vitro* cell binding studies of chimeric MAbs have indicated the possible retention of the antibody-antigen binding properties of the original murine antibodies, and tissue distribution studies in mice have shown a similar tumor-to-tissue ratio to that of murine MAb (Colcher, et al., 1989; Brown, et al., 1987; Saga, et al., 1990). Another development in respect to

chimeric antibody is called reshaped, CDR (complementarity-determining regions)-grafted or 'humanized antibodies' (Co, et al., 1991). Instead of the whole V domain of a murine antibody, only the CDRs which are critical to the antigen binding from a rodent antibody are introduced into a human V-region framework. The humanized antibodies produced by this method were reported to be less immunogenic in humans (Co, et al., 1991).

The production of bispecific monoclonal antibodies has generated much interest due to their potential applications. The normal IgG molecule is bivalent and monospecific, containing two identical antigen binding sites. Bispecific antibody molecules, in contrast to the conventional ones, possess two different antigen binding sites per molecule. The use of bispecific antibodies can eliminate the need for chemically coupling the desired reagent, such as drugs and chelating agents to antibodies. The bispecific antibodies were used as agents for the selective immobilization of enzymes (Brennan, et al., 1985). The use of radiolabeled bispecific antibodies in tumor imaging was also reported (Doussal, et al., 1990). Bispecific antibodies can be produced by fusion of two hybridomas, or of one hybridoma with spleen cells from an immunized animal. The hybrid hybridomas generated by this method secreted predefined bispecific MAbs as well as monospecific ones (Milstein, et al., 1983). Purification of the antibody mixture to fractionate the bispecific and monospecific MAbs is required. A new technique in preparation of bispecific antibodies by chemical reassociation of monovalent fragments of IgG molecule was developed (Brennan, et al., 1985). This method was reported to produce bispecific antibodies in high yields and free of monospecific contaminants.

Since the uptake of circulating MAb to the tumor is dependent upon its vascularity and permeability, small sized fragments of the whole MAb were used (Buchegger, et al., 1983; Brady, et al., 1987). Meanwhile, it was reported that the Fc region of the antibody molecule was most likely to trigger allergic responses and lower HAMA response was observed when fragments of MAb were used instead of intact IgG (Keenan, et al., 1985; Buraggi, et al., 1991).

The enzyme pepsin acts adjacent to the disulfide bonds bridging the H chains

(Fig. 2.1), cleaving off most of the Fc region and leaving the two Fab fragments bound together in a divalent structure called $F(ab')_2$ fragment. On the other hand, the enzyme papain digestion (Fig. 2.1) resulted in the formation of two monovalent Fab fragments and an intact Fc portion (Keenan, et al., 1985). The comparison of the radioiodinated MAbs and their fragments *in vivo* in tumor bearing nude mice indicated that an increase of tumor/tissue ratio with MAb fragment was in part due to their more rapid elimination, the absolute concentration of fragments in tumors was also decreased as compared to intact MAbs, but to a lesser degree than their concentration in normal tissue (Buchegger, et al., 1983).

Recent advances have been the development of single chain antigen binding protein (SCA) by utilizing cloning of Ig gene technology (Schlom, et al., 1991). SCAs with a molecular weight of only 25,000 Daltons are composed of a variable light chain amino acid sequence of an Ig tethered to a variable heavy chain sequence by a designed peptide. The *in vitro* binding studies showed that ^{125}I-labeled B6.2 IgG fragment Fab and SCA competed similarly (Schlom, et al., 1991). The *in vivo* biodistribution studies in tumor bearing mice showed higher tumor/normal tissue ratios for SCA than for Fab fragment (tumor/blood, 6.24, 7.26 and 3.37, 5.26 at 6 and 24 hours PI for SCA and Fab respectively) (Schlom, et al., 1991). Their data also showed that the kidney uptake of SCA was largely reduced compared to those of Fab and $F(ab')_2$.

Recently a novel approach for the detection of necrotic lesions of tumors has been developed (Epstein, et al., 1988; Chen, et al., 1989). The hypothesis is based on the well known phenomenon that in contrast to normal tissues, many malignant tumors undergo degeneration and cell death with formation of large areas of necrosis. The loss of membrane integrity permits macromolecules to freely enter the cell cytoplasm (Chen, et al., 1989). It is hypothesized that MAbs to an intracellular antigen, which is an integral structural component and is retained by degenerating cells, may be used to target a wide range of MAbs against human malignancy. Data presented by Epstein, et al. (1988) have demonstrated feasibility of imaging four different histological types of human cancer in a nude mice model using two MAbs,

TNT-1 (IgG_{2a}) and TNT-2 (IgM), against an insoluble intranuclear antigen (histone). A comparative autoradiographic study evaluated the differential intratumor localization of two MAbs, Lym-1 which is directed against a cell surface antigen and TNT-1 against intracellular antigens (Chen, et al., 1990a). Evidence was presented to indicate that MAb Lym-1 accumulates at the periphery of the target tumor while TNT-1 progressively concentrates in the center of the tumor where binding to its nuclear antigen occurred. Recent preliminary clinical results using 131-I-labeled $F(ab')_2$ of TNT-1 suggest that TNT is preferentially localized in the necrotic tissue of the tumor with only little binding to normal tissues (Chen, et al., 1990b).

2.3 Asialoglycoprotein and Carbohydrate Conjugates and Their Possible Role in Immunoscintigraphy

2.3.1 Sialic Acid

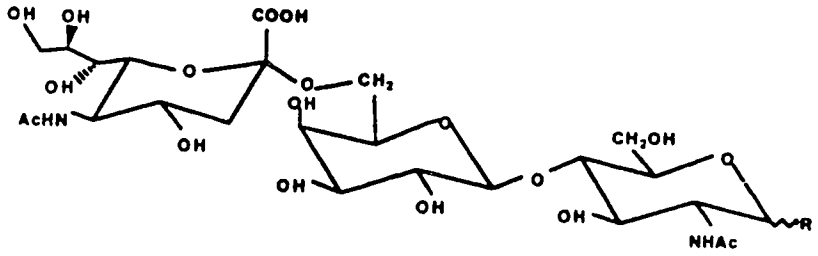
2.3.1.1 General Properties of Sialic Acid

Sialic acid occurs in α-glycosidic linkage as a component of a number of oligosaccharides, polysaccharides, and glycoproteins. A monoclonal antibody is also a glycoprotein. The name sialic acid was first used by Blix, Gottschalk and Klenk (cited by Schauer, 1982b) and this carbohydrate group is composed of N- and O-acyl derivatives of neuraminic acid. Sialic acid usually exists in the terminal position in oligosaccharide chains. One of the common sugar sequences (trisaccharide) incorporating the terminal sialic acid presented in serum glycoproteins is shown in Fig. 2.2. Sialic acid (N-Acetylneuraminic acid, NANA) is followed by a galactose (Gal) group and then a N-acetylglucosamine (GlcNAc)(Krohn, et al., 1982). Other linkages of sialic acids in complex carbohydrates have been reported as well (Schauer, 1982a; Watanabe, et al., 1979).

A class of enzymes which are involved in the cleavage of α-glycosidic bonds of sialyl residues in oligosaccharides and glycoproteins is known under the term of sialidases. These are the most important enzymes for initiation of the catabolic metabolism of sialoglycoconjugates and sialo-oligosaccharides by the hydrolytic

release of the α-glycosidically bound sialyl residues. Enzymic hydrolysis of sialic acid residues, using sialidases, was employed for the isolation of sialic acid, the structural investigation of sialic acid-containing glycoproteins and cell membranes (Schauer, 1982b).

Another type of enzymes, sialyltransferases, such as ß-D-galactoside-α-(2,6)-sialyltransferase, 2-acetamido-2-deoxy-α-D-galactoside-α-sialyltransferase, etc, are required for the biosynthesis of specific sialic acid linkages observed in glycoproteins (Beyer, et al., 1979; Paulson, et al., 1977). Thus, the appearance and disappearance of specific sialic acid residues on a particular polysaccharide or glycoprotein is regulated by the presence or absence of enzymes which induce such action.



NANA-α(2,6)-Gal-ß(1,4)-GlcNAc

Fig. 2.2 A common structure of the terminal trisaccharide of oligosaccharide moieties on plasma glycoproteins

2.3.1.2 Anti-Recognition Effect of Sialic Acid

The anti-recognition effect of sialic acid in the liver uptake of glycoproteins was first described by Morell, et al. (1968) following the discovery that radiolabeled asialoceruloplasmin was accumulated by parenchymal cells of the rabbit liver and cleared from the blood circulation rapidly upon intravenous (iv) injection. Gregoriadis, et al. (1970) later showed that more than half of the radioactivity was recovered within minutes after the administration of ^{64}Cu and ^{3}H labeled desialylated ceruloplasmin, principally in hepatocytic lysosomes. From the facts that

denatured plasma proteins are found to be deposited only in Kupffer cells, and that native serum albumin as well as desialylated ceruloplasmin are associated with the hepatocytes rather than Kupffer cells, existence of specific receptor sites in the liver which have a physiological significance have been suggested (Ashwell and Morell, 1974).

The initial demonstration of this recognition phenomenon using asialoceruloplasmin, and the extension of these studies to a variety of other serum glycoproteins such as fetuin, orosomucoid, haptoglobin, thyroglobulin, glycophorin, immunoglobulin G, etc, established the generality of the phenomenon (Ashwell, et al., 1982; Schauer, 1982a; Harford, 1982). Thus, it was suggested that sialic acid, which usually occurred as a terminal residue in most glycoproteins, was responsible for the masking the D-galactosyl residue, thus protecting the survival of these molecules in the circulation. Removal of the terminal sialic acid of most mammalian plasma glycoproteins exposes galactose as a nonreducing sugar terminus and results in rapid endocytotic transfer of these proteins from the circulation into hepatocellular lysosomes where catabolism occurs (Stockert, et al., 1980).

The protecting effect of sialic acid is further verified by enzymic replacement of the missing sialic acid of the desialylated glycoprotein (Hickman and Ashwell, 1970). When the sialic acid residues of desialoceruloplasmin were restored using the sialyl transferase from the rat liver in the presence of CMP-N-acetylneuraminic acid-1-^{14}C, 50% of the labeled protein survived in the serum of rabbits with a normal half-life of 54 hours.

Another important aspect is that sialic acid residues may mask structures which later appear as tumor antigens. For example, Thomsen-Friedenreich (T-F) antigen is normally in a cryptic or masked form with terminal sialic acid links to Gal residues. Unmasked or free T-F antigen is reported in a variety of animal and human carcinomas, and is thought to arise as a result of malignant transformations during differentiation. There exists a number of review papers in this area (Uhlenbruck, et al., 1981; Noujaim, et al., 1983; Springer, 1984).

2.3.1.3 Sialic Acid as a Component of the Liver Receptor

Paulson, et al., (1977) reported the reactivation of asialo-rabbit liver binding protein by resialylation with ß-D-Galactoside-α(2,6) sialyltransferase. The rabbit liver membrane protein which binds galactosyl residues of asialo-plasma glycoproteins loses its binding capacity after enzymatic removal of sialic acids from its carbohydrate moieties. Reincorporation of the sialic acid which was previously removed resulted in a restoration of 80% of the original binding activity.

This was also shown when complete desialylation of erythrocytes, followed by specific incorporation of sialyl residues with the aid of the ß-D-galactoside-(2,3)-sialyltransferase, led to the restoration of the original binding capacity of the erythrocyte to polyoma virus (Paulson, et al., 1979).

2.3.2 Carbohydrate-Specific Receptors in Liver

The carbohydrate-specific receptors in the liver were initially described after the finding that the injection of desialylated ceruloplasmin into rabbits resulted in the prompt clearance of the protein from the circulation under conditions in which the fully sialylated protein survived for days (Morell et al., 1968). With the successful separation, purification and characterization of rabbit, rat, human and mouse liver carbohydrate receptors, the binding properties of these mammalian receptors have been elucidated (Baenziger, et al., 1980; Weigel, 1980; Sarkar, et al., 1979; Bystrova, et al., 1985). It has been reported that there may be several hepatic receptors capable of recognizing and binding specific nonreducing carbohydrate termini. An extensive review in this area is given by Ashwell (1982) and Harford (1982).

2.3.2.1 Galactose/N-Acetylgalactosamine (GalNAc) Specific Receptor

Because the sialic acid residues preceeded galactose on carbohydrate termini of most serum glycoproteins, extensive studies of desialylated serum glycoproteins implicated that a galactose specific receptor was present in the liver. The newly exposed galactose residues were further proven to be the recognition determinant

by the findings that treatment of asialoceruloplasmin with galactose oxidase or ß-galactosidase (Morell, 1968) or enzymatic replacement of the missing sialic acid residues (Hickman, 1970) recovered the survival time of the protein in the circulation.

A study using desialated antibody (Winkelhake, et al., 1976) showed that removal of terminal sialic acid resulted in rapid blood clearance and enhanced localization in the liver, whereas subsequent removal of penultimate galactose residues returned the clearance to near normal levels. On the other hand, removal of subpenultimate GlcNAc residues resulted in an intermediate survival value between asialo- and asialo-agalacto-antibodies.

Further evidence was obtained from studies on isolated hepatocytes and in the perfused liver as well as from a detailed examination of the structural requirements for ligand binding (Weigel, 1980; Sarkar, et al., 1979; Regoeczi, et al., 1977; Bystrova, et al., 1985; Stowell, et al., 1980). It was reported that freshly isolated rat hepatocytes bound ^{3}H-asialo-orosomucoid in a sugar-specific and a calcium-dependent manner (Weigel, 1980). In another experiment the rabbit hepatic lectin was isolated and immobilized on Sepharose gel for examination of its binding site by a competitive binding assay (Sarkar et al., 1979). The results of these investigations showed that the binding site was relatively small, involving a terminal nonreducing saccharide chain containing an axial hydroxyl on carbon 4 and extending to at least part of the penultimate sugar residue. Methyl glycosides of N-acetylgalactosamine were more effective inhibitors of ligand binding than methyl glycosides of galactose.

The preference for GalNAc over Gal for the receptor site was reported in ealier studies (cited from Harford, et al., 1982). The results indicated that group A erythrocytes (having GalNAc residues as determinants) were more sensitive to agglutination by the rabbit lectin than group B erythrocytes (with Gal determinants). Similar results were obtained in studies of isolated human hepatic lectin (Baenziger, et al., 1980) and of the rat asialoglycoprotein receptor from isolated hepatocytes (Harford, et al., 1982).

Another experiment was carried out by Stockert, et al. (1980) using an antibody against purified rat hepatic lectin. The infusion of this antibody into a portal vein of perfused rat liver prior to injection of radiolabeled asialo-orosomucoid lowered the rate of influx of asialo-orosomucoid into liver by over 80%. The results demonstrated that interaction with a specific cell surface lectin is essential for removal of asialo-orosomucoid from the circulation.

Studies of the chemical and physical properties of purified hepatic membrane lectins of rabbit, rat, mouse and humans indicated that they were quite similar in terms of the glycoprotein nature, had sialic acid as a component, and showed a calcium dependency (Harford, 1982; Ashwell, 1983).

2.3.2.2 Mannose/GlcNAc Specific Receptors

Stockert, et al. (1976) found that removal of galactose from asialo-orosomucoid, which results in exposure of terminal GlcNAc residues, was also accompanied by rapid hepatic uptake. Significantly, the clearance of this derivative was not inhibited by the simultaneous injection of asialo-orosomucoid. A recognition system, specific for GlcNAc, was thus postulated. A surprising observation by Achord, et al. (1977) demonstrates that the clearance of agalacto-orosomucoid (GlcNAc-terminal) from rat plasma is inhibited by infused mannans or mannose alone. Gal-terminal ligands are inert in this system.

Extension of these studies with RNase B, a naturally occurring mannose-terminal glycoprotein, and with RNase A, a neoglycoprotein to which mannose is attached chemically, provided further information on the participation of mannose in this hepatic uptake system. Studies of the binding sites in the liver, at several laboratories, by histological examination, direct binding assays, and by electron microscopic autoradiography showed that Kupffer cells were the principal loci of the uptake (Ashwell, et al., 1982). Thus, cell type localization was a second parameter of differentiation between this system and that for the uptake of asialoglycoproteins.

Carbohydrate-mediated clearance of antibody(IgM)-antigen complexes by mannose specific liver receptors from the circulation was described by Day, et al. (1980). Anti-BSA antibody was produced after immunization of rats with BSA for

a 12 day period. Soluble IgM-^{125}I-BSA complexes were rapidly cleared from the circulation of nonimmunized rats, and clearance was inhibited by ovalbumin, but not asialofetuin. The immune complexes were recovered primarily in hepatic nonparenchymal cells and in other organs of the reticuloendothelial system. The data from *in vitro* binding experiments suggested that antigen-induced conformational changes can result in exposure of high mannose oligosaccharides on IgM which signalled the clearance of soluble immune complexes from the circulation.

Recently, the evidence for specific recognition sites on the A chain immunotoxins that mediate rapid clearance of the antibody-ricin-A-chain immunotoxin was provided by Bourrie, et al. (1986). These authors found that neither the properties of the antibody moiety nor the nature of the linkage of ricin A-chain to antibody was responsible for the disappearance of immunotoxin from the plasma. It was the mannose residues on the ricin A-chain moiety that were specifically recognized by the liver cells.

2.3.3 Carbohydrate Conjugates (Neoglycoproteins)

Instead of removing terminal sialic acid groups to expose the essential galactose residue, an analog was produced by covalently conjugating sugars to proteins. A neoglycoprotein was defined by Stowell and Lee (1980), as a glycoprotein produced chemically by the covalent attachment of sugars to proteins that may or may not already contain naturally-bound carbohydrates. Neoglycoproteins have been used to study the roles of the binding of carbohydrates with lectins. The advantage of this approach is that relatively large amounts of neoglycoproteins in which the structures of the carbohydrates are well defined can be obtained. It is a task that may be more difficult if naturally occuring glycoproteins are used. An extensive review on this subject was presented by Stowell, et al. (1982) and Lee, et al. (1982).

2.3.3.1 Preparation of Neoglycoproteins by Chemical Methods

A number of criteria need to be considered when choosing the methods of preparation since utilization in biological research is usually the ultimate objective of the preparation of neoglycoproteins. These considerations include mildness of the reaction (preservation of the native structures), stability of the linkage and controlability of the number of residues.

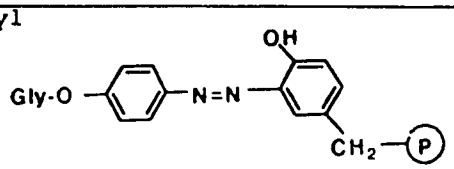
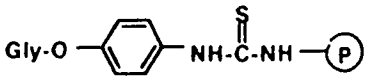
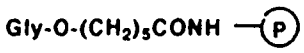
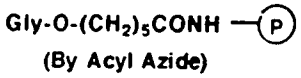
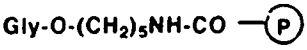
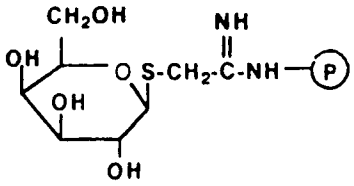
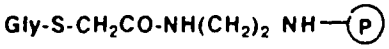
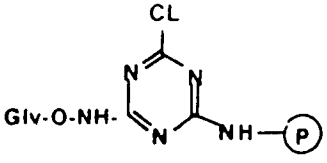
Table 2.1 gives a summary of some of the common approaches utilized in the preparation of neoglycoproteins. Among them, diazo coupling is considered as nonspecific and frequently inactivates enzymes or precipitates proteins. The azophenyl group was found to be immunogenic when injected into animals as well.

The amidation approach of attaching carbohydrates to proteins involves an amide-bond formation that is quite stable in physiological conditions. However amidation using water-soluble carbodiimide is not efficient. Polymerization occurred as a result of intra- or inter- molecular cross-linkage (Blair, et al., 1983; Lonngren, et al., 1976; Lee, 1982). Another amidation method frequently used is the acyl azide method (Lemieux, et al., 1977). This method has been used extensively for the preparation of synthetic antigens containing mono- to tetrasaccharides.

Modification of amino groups on proteins with imidate results in the formation of amidino derivatives. This method was reported to have a lesser effect on the biological properties of the protein because of the retention of the cationic properties of the original amino groups (Lee, 1982).

Carbohydrates can also be attached to amino groups of proteins by reductive amination of reducing sugars as well (Gray, 1974; Schwartz, et al., 1977; Roy, et al., 1984). But because of the low concentrations of free aldehyde form in sugar solution, when reducing oligosaccharides are used in this reaction, the reaction time is usually several days or longer. The use of a higher temperature (37^{0}C), pH 8-9, and the addition of borate were reported to improve the procedure (Roy, et al., 1984). It was noticed that the ring structure of the reducing sugar was eliminated in this type of reaction and that this could present a problem if this structure is

Table 2.1 Methods used in the preparation of neoglycoproteins

Methods	Functional group on proteins	Coupling products	References
Diazo	tyrosyl, lysyl histidyl		Means, 1971
Isothio-cyanates	amino amino		McBroom, 1973 Smith, 1978
Amidation	amino		Krantz, 1976 Lonngren, 1976
	amino		Lemieux, 1977
	carbonyl		Moczar, 1976
Amidi-nation	amino		Lee, 1976 Stowell, 1980
Reductive amination	amino		Means, 1968 Gray, 1974 Lee, 1980
Cyanuric chloride	amino		Chaudhari, 1972

critical for biological activity. The reaction rate and yield are increased considerably when synthetic glycosides having an aldehydo group are used and $NaBH_3CN$ is chosen as a reducing agent in the reductive amination (Lee and Lee, 1980).

Utilization of synthetic glycosides containing suitable functional groups in the preparation of neoglycoproteins affords more flexibility and often better controlability and efficiency. A series of glycosides of different sugars may be prepared with the same aglycon, and different aglycons may be attached to the same sugar for investigation of the influence of the sugar structures on interaction with receptors or other biological behaviour. In terms of preservation of biological properties of the proteins under investigation it was recommended that amidation and reductive alkylation were the most suitable methods (Lee and Lee, 1982). In practice, the choice of the modification approach is determined by the nature of carbohydrates to be attached, as well as the nature of proteins. The availability of reagents and experimental conditions must be considered. The intended use of neoglycoproteins may also determine the strategies employed. A variety of strategies will provide more options that are suitable for different purposes. One of the severe limitations is the difficulty in the technology of preparing derivatives of saccharides.

2.3.3.2 Influence of Ligand Structures on Their Interaction with Receptors

Neoglycoproteins have proved to be powerful tools for investigating the carbohydrate-mediated binding and cellular uptake of glycoconjugates. The binding experiments are conducted either on liver membrane protein solutions or Sepharose-immobilized liver receptors. Most of studies are based on the assay developed by Van Lenter and Ashwell (1972), and Hudgin, et al. (1974). The relative inhibitory power (RIP), which was defined as the amount of unlabeled asialo-orosomucoid causing 50% inhibition of ^{125}I-asialo-orosomucoid binding divided by the amount of test compound causing 50% inhibition of ^{125}I-asialo-orosomucoid binding was used to compare quantitatively the inhibitory power of various neoglycoproteins. The binding of neoglycoproteins by the hepatic lectins was found to be many orders of magnitude stronger than the binding of free monosaccharide

(Harford, et al., 1982). The results also indicated that neoglycoproteins having clusters of galactose residues per point of attachment were much more tightly bound by asialoglycoprotein receptors than those containing an equivalent number of galactose residues attached as monosaccharide units (Kawaguchi and Lee, 1980). Similar results were described by Baenziger, et al. (1980) when they were investigating the binding properties of isolated human hepatic lectin. They suggested that this effect was most likely due to the simultaneous binding at two sites rather than to a statistical effect of increased number of residues.

Clustering of terminal sugars could be provided by a branched structure of carbohydrate chains. In the case of orosomucoid molecule, 5 branched-chain carbohydrate structures account for 40% of its weight. This arrangement may explain the avid binding to membranes and isolated receptors as well as the extremely rapid removal of asialo-orosomucoid from the circulation (Harford, et al., 1982). In a specturm of binding affinities, asialo-orosomucoid was shown to have the highest affinity. On the other end of the spectrum of binding affinities was asialotransferrin. In the initial survey by Morell, et al. (1971), asialotransferrin clearance could not be distinguished from that of native transferrin. When the observation was extended to several days, human asialotransferrin was found to disappear from circulation at a faster rate than the original transferrin (Regoeczi, et al., 1974). Transferrin was reported to exhibit carbohydrate heterogeneity, having biantennary and triantennary branches. Enhanced binding for triantennary structure and intact asialotransferrin containing two biantennary glycans were reported (Hatton, et al., 1979). However, the binding and endocytosis processes were suggested to be of greater complexity. Some of these processes are still unknown.

2.3.3.3 Applications in Radioimmunoimaging and Therapy

Carbohydrate-modified antibodies were examined for possible applications in radioimmunotherapy. A technique for treatment of ovarian carcinoma or other malignant cells confined to the peritoneal cavity by intraperitoneal (ip) injection of galactosylated antibody was reported by Mattes et al. (1987). In normal mice,

transfer of radioiodinated antibodies from the peritoneal cavity to blood was found to be very fast. The presence of ascitic fluid in mice greatly delayed the rate of transfer. Injection of galactose-conjugated IgG resulted in rapid clearance via a hepatic lectin after transfer of the IgG molecule to the circulation from the injection site. Moreover, when intravenous administration was accompanied by injection of a competitive hepatic lectin binding inhibitor to control the clearance rate, optimal uptake by the target tissues was obtained (Mattes, M. J. European patent 88308512.8, 1988; Ong, et al., 1991). An interesting observation noted from Mattes's experiment is that neuraminidase treatment of all major mouse immunoglobulin (Ig) classes and subclasses, including IgM, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, and IgA did not result in their rapid blood clearance, although the similar treatment of fetuin was effective. Similarly, in the case of transferrin, it was originally observed that neuraminidase treatment did not result in rapid clearance from the circulation. However, other laboratories have shown that in certain cases desialylated rabbit IgG was cleared by this mechanism (Winkelhake and Nicolson 1976).

The enhanced clearance of a plant lectin by a chemically glycosylated antibody generated against plant lectin was reported by Kojima et al. (1989, 1990). Antibody-antigen (plant lectin in this case) complexes were cleared very fast; the remaining radioactivity in blood was observed to be largely reduced within 15 minutes upon injection of the chemically galactosylated antibody. As a result, the tumor-to-blood ratio was significantly improved from 1.66 to 3.35 since the binding of the plant lectin to tumor in the presence of antibody was not affected (Kojima, et al., 1990). The results of these investigations also showed that the modification process by amidination did not affect the antibody activity and the clearance rate. However, they also demonstrated that the tumor-to-blood ratio depended on the number of sugar residues on the antibody molecule. The utilization of a second antibody (IgG) directed against an immunoradiopharmaceutical was reported in an attempt to reduce the background radioactivity in blood (Goldenberg, et al., 1987) as well. The induced conformational changes of the antigen by exposure of galactose residues in the antibody-antigen complexes may contribute to increased hepatic

access and then mediate the binding and uptake on hepatic cells (Thornburg, et al., 1980).

2.4 Periodate Oxidation of Glycol Residues

Oxidation with periodate, resulting in 1,2-glycol scission is one of the most widely used reactions in carbohydrate chemistry since its discovery by Malaprade in 1928 (cited from Bobbitt, 1956). Mild conditions of the reaction, a high degree of selectivity when applied under proper conditions, and a simplicity of application have contributed widely to the use of this reaction. Analytical procedures are available for following the reaction course. The knowledge that specific groups on carbohydrates may produce certain stable end-products also made possible the elucidation of unknown structures of such compounds. Extensive reviews in this area are given by Bobbitt (1956); Blair, et al. (1983); and O'Shannessy, et al. (1985, 1987).

2.4.1 Proposed Mechanism

Univalent, nonhydrated metaperiodate ion (IO_4^-), which prevails between approximately pH 3 and 8 at 25°C (Taylor, et al., 1955), is responsible for the vicinal hydroxyl cleavage. The mechanism of periodate cleavage of glycols has been suggested to involve the formation of cyclic periodate ester (Price, et al., 1942; Ghose, 1988; Chinn, 1971; Vollharot, 1987). Fig. 2.3 gives the proposed mechanism of periodate oxidation of glycols (Vollharot, 1987).

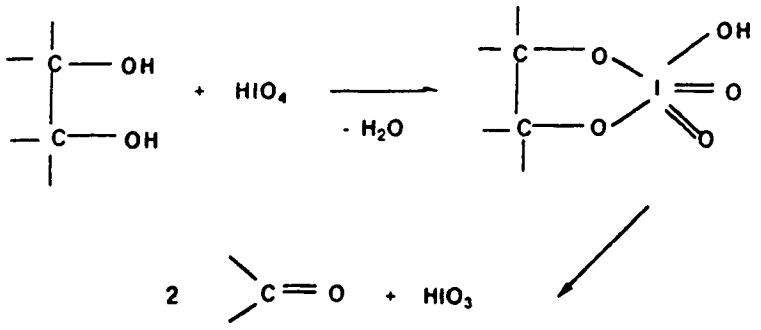


Fig. 2.3 Proposed mechanism of periodate oxidation of glycols

With cyclic α-glycols containing less than 10 carbon atoms, the cis isomers are oxidized more rapidly than the trans ones (Price, et al., 1942; Chinn, 1971; Bobbitt, 1956). The cis isomer of cyclohexane-1,2-diol is approximately 30 times more reactive than the trans one (Bunton, et al., 1965). The oxidation of those methyl aldohexopyranosides having cis vicinal hydroxyl groups, such as methyl α-D-mannopyranoside and methyl α-D-galactopyranoside was shown to proceed faster than those of methyl α-D-glucopyranosides having trans diols (Bobbitt, 1956). These studies support the proposed mechanism of formation of a cyclic, rather than an open-chain intermediate since most of these less reactive diols have geometries that prevented the formation of a cyclic periodate ester.

Rothfus and Smith (1963), in a detailed experiment (later called Smith degradation) (Lenten and Ashwell, 1971) with glycopeptides from human gamma-globulin which served as a model molecule, established some general rules for elucidation of carbohydrate structures in glycoproteins by the periodate oxidation method. In the case of periodate oxidation of a glycoprotein having a known structure of the carbohydrate moiety, the rules for theoretical considerations of the possible periodate oxidation are these: the terminal, non-reducing monosaccharide, as well as those pyranosidically-linked neutral sugars which are not substituted at carbon 3 may be subject to periodate oxidation. Using these rules one can predict the number of monosaccharides which may be vulnerable to periodate oxidation. Fig. 2.4 shows some of the monosaccharides commonly occurring in glycoproteins.

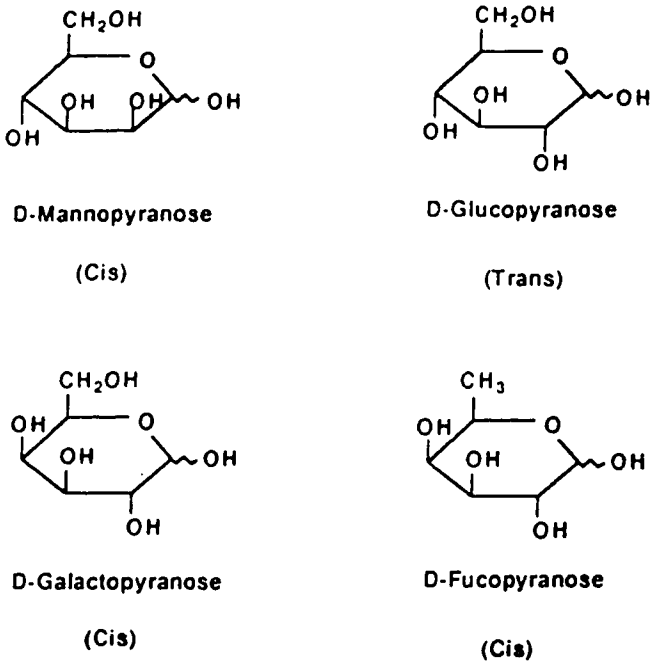


Fig. 2.4 Structures of monosaccharides commonly occurring in glycoproteins

2.4.2 Effect of Reaction Conditions (Concentration of Oxidant, pH, Temperature and Light)

2.4.2.1 Effect of Concentration of the Oxidant

Sodium periodate is usually used as an oxidizing agent in periodate oxidation. Under suitable mild conditions oxidation of glycoproteins can be directed to terminal sialic acid only. Quantitative conversion of terminal sialic acids of ceruloplasmin and orosomucoid to radioactive 7-carbon analogues was achieved by controlled periodate oxidation, with 10 mM of sodium periodate, at 0°C for 10 min, and then tritiated borohydride reduction (Lenten, and Ashwell, 1971). A similar experiment was performed by Gahmberg, et al. (1977) using low concentration of sodium periodate, and selectively labeling the sialic acid residues of sialoglycoproteins on a cell surface. Weber, et al. (1975) demonstrated that following mild periodate oxidation (5 mM of H_5IO_6, 0°C for 30 min) dansylhydrazine, as a fluorescent label, could be introduced mainly into the sialic acid residues of the

glycoproteins. More severe conditions resulted in the oxidation of sugars other than sialic acids and lead to the destruction of the terminal, non-reducing sugar residues as well as those pyranosidically linked neutral sugars within the oligosaccharide chain (Lenten and Ashwell, 1971). The periodate concentration used in the oxidation of glycoproteins is generally in the range of 0.5-100 mM. The concentration of periodate greater than 500 mM was reported to cause nonspecific oxidation of the protein molecule (Ghose, et al., 1988; Moe, et al., 1951).

2.4.2.2 Effect of pH and Buffer Systems

The periodate oxidation rate of ethylene glycol was found to attain a broad maximum between pH 2.5 and pH 6 (Taylor, et al., 1955). While the most rapid oxidation of simple amino alcohols, such as serine and threonine occurred at a pH of 7.5 (Bobbitt, 1956; Winnick, 1942; Boyd, et al., 1942). It was reported that phosphate-buffer solutions can cause erroneous results and should be avoided in periodate oxidation of carbohydrates (Bell, et al., 1949, 1950; Greville, et al., 1952). The pH values outside the range 3 to 5 are not recommended in the application of this reaction to glycoproteins.

Antibodies are natural glycoproteins and it has been known that the carbohydrate portion is located almost exclusively on the Fc region that is not involved in the antibody-antigen binding activity. This portion inevitably became the target of chemical manipulation of immunoglobulins. In the investigation of the favorable conditions for oxidation of immunoglubulin G, Murayama, et al. (1978) examined the effect of different buffer systems and pH values. The conditions used by these investigators were: 0.1 M acetate buffer, pH 4.5, 5.5 and 6.5; Atkins-Pantin buffer, pH 7.5, 8.5 and 9.5; 0.1 M phosphate buffer, pH 7.0 and 8.0; and 0.1 M carbonate-bicarbonate buffer, pH 9.0 in 20 mM of $NaIO_4$ in the dark and at room temperature (25^{0}C) for 30 min. The data showed that antibody activities were maintained when the reaction was carried out at an antibody concentration of 20 mg/mL in acetate and Atkins-Pantin buffers. When the oxidation was performed at pH 4.0, the product eluted from a Sepharose 6B column was at the same position

as that of native IgG. When the buffer pH was 6.5 a peak appeared at void volume, indicating possible polymerization. Following the recommendation given in this last paper, acetate buffer, pH 4 to 5.5 has been the most commonly used condition for periodate oxidation of antibodies by other researchers (Ghose, et al., 1988; Hoffman, and O'shannessy, 1988). Imidazole buffer was found unsuitable for periodate oxidation due to the reported presence of a reducing agent as an impurity (Ghose, et al., 1988).

2.4.2.3 Effect of Temperature and Light

It has been reported that a more random type of oxidation occurred at high temperatures and, to suppress nonspecific oxidation, temperatures at 0-4°C were recommended (Bobbitt, 1956). It was also reported that aqueous sodium periodate was slowly decomposed in the presence of light, to yield sodium iodate and ozone. Oxidation reactions, usually carried in the dark, were found to mitigate undesired side-products (Bobbitt, 1956).

2.4.2.4 Specificity of the Reaction

One potential problem with periodate oxidation of glycoproteins is the oxidation of some of the amino acids. It was reported that cysteine, cystine, methionine, tryptophan, tyrosine and histidine, serine and threonine would be oxidized if they occurred as the terminal residues on glycoproteins (Clamp and Hough, 1965). However, studies with intact glycoproteins suggested that amino acids were less susceptible to periodate oxidation when present in a peptide chain (Krotoski and Weimer, 1966). One hundred twenty mM of periodic acid at 25°C-26°C for 6-12 hours were required for oxidation of tyrosine and sulfur containing amino acids in human plasma orosomucoid while the other amino acids remained intact. Therefore, the oxidation of amino acids with periodate appeared to be a problem only under extensive oxidation at room temperature for several hours. The more commonly used conditions for periodate oxidation of antibodies and glycoproteins, at 0°C for 0.5 to 1 hour, should minimize the potential oxidation of

amino acid backbones of the glycoproteins.

It has been shown that reaction of biotin hydrazide with either polyclonal antibodies or MAbs was dependent on prior oxidation of antibodies with $NaIO_4$ (O'Shanessy and Quarles, 1985). In addition, deglycosylation of the biotinylated antibodies with endoglycosidase F removed the biotin label, indicating that the label was on the N-linked oligosaccharides. A number of authors have also shown that treatment of immunoglobulins with neuraminidase prior to labeling decreased the amount of incorporation by approximately 90% during a controlled oxidation directing a label to sialic acid residues (O'Shannessy and Quarles, 1987). Taken together, labeling of immunoglobulins can be made specific for the carbohydrate moieties under appropriate conditions.

The more critical issue, however, was the effect of periodate treatment on antibody activity toward its antigen. Several reports have shown that there is no measurable effect on antibody affinities by the labeling of oligosaccharide moieties of immunoglobulins under mild oxidations with $NaIO_4$ (Blair, et al., 1983; Chua, et al., 1984; O'Shannessy, et al., 1985; Rodwell, et al., 1986; Hoffman, et al., 1988). A review article discussed the presence of two moieties on immunoglobulins which may provide linkage sites not likely to interfere with the antigen binding (Blair, et al., 1983). One such moiety is the carbohydrate group which is usually present in the CH2 region of heavy chain and only occasionally in the V region. The other linkage site that is likely to allow retention of the antibody specificity can be provided by free sulfhydryl groups formed from reductive cleavage of interchain disulfide bridges in immunoglobulins. As discussed in the following section, direct labeling of antibodies using ^{99m}Tc utilizes sulfhydryl groups.

It is worth noting when dealing with antibodies, especially with monoclonol antibodies, that sensitivity of antibodies to oxidation with $NaIO_4$ varies. One of the monoclonal antibodies tested was sensitive to oxidation when labeled with biotin hydrazide and a successful conjugation could be achieved after oxidation at 0^0C but not at room temperature (O'Shannessy and Quarles, 1985). Hoffman, et al. (1988) reported that goat antibodies could be oxidized at pH 4.5 and still retain full

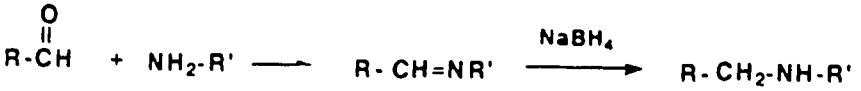
biological activity, while rabbit antibodies are partially inactivated under the same conditions as determined by dot-blot analysis. However rabbit antibodies were stable when coupled to hydrazide gels at pH 4.5 if the oxidation had occurred at a higher pH. In general, a lower pH had resulted in a better conjugation yield in their experiments.

2.4.3 Condensation of Periodate Oxidized Vicinal Diols with Hydrazine/Hydrazide or Amino Compounds (Schiff Base Formation)

2.4.3.1 General Aspects of Condensation Reaction of Aldehyde and Amino Compounds

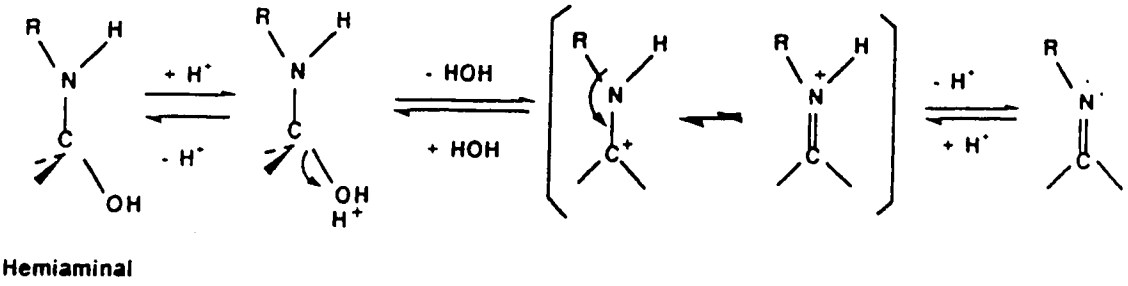
Two basic type of condensation reactions are given in Fig 2.5. The first type is the condensation of amino compounds with aldehydes and the formation of imine (Schiff base). Imine formation starts from a nucleophilic addition to a carbonyl group followed by a rapid proton transfer and results in production of hemiaminal. The next step is water elimination from hemiaminal which is the rate-limiting step. Dehydration of the hemiaminal begins with the protonation of the hydroxyl group, while the protonation of more-basic nitrogen just leads back to the carbonyl compound (Fig. 2.6). The overall reaction obeys the following rate law: rate = k [carbonyl] $[H^+]$ $[RNH_2]$. Although the reaction is acid-catalyzed at moderate pH, at low pH the rate actually diminishes with an increasing acid concentration because the nucleophilicity will be reduced if the nitrogen base is protonated. The concentration of the free nucleophile is inversely related to acid concentration. Since the concentration of unprotonated nitrogen base is so low in low pH solutions, nucleophilic addition may become a rate-limiting step (Cordes, et al., 1962; Streitwieser and Heathcock, 1976; Vollhardt, 1987).

Type 1



Type 2

Fig. 2.5 Condensation of an aldehyde group with a hydrazine/hydrazide or an amino compound



Hemiaminal

Fig. 2.6 Dehydration of hemiaminal formed by amino and aldehyde groups

Simple alkylamines have pK_a in the range of 10-11. High basicities allow them to become readily protonated and consequently diminish the concentration of free amines needed in nucleophilic reaction. This also increases the chance of protonation of the nitrogen in the hemiaminal dehydration step (Fig. 2.6) causing the imine formed to revert back to the starting carbonyl compound. For these reasons the imine formed from the reaction of a simple aliphatic amine with an aldehyde is not stable and usually requires reduction to a secondary amine. This is the principle of the reductive amination used in protein modifications.

$NaBH_4$ was used as a reducing agent in previous reductive aminations. However, the success of the reaction was conformed upon the discovery of the better selectivity of $NaCNBH_3$ in reducing imine double bonds rather than carbonyl

groups (Jentoft, et al., 1979). The electron-withdrawing cyanide group decreases the ability of borohydride ion to deliver H^- to the functional group being reduced. Therefore, cyanoborohydride is more discriminating in its reducing ability. In contrast to sodium borohydride which requires a pH 9 for aqueous media, cyanoborohydride is also fairly stable in acid media (pH 6) and is thus ideal for the reductive amination of proteins.

A second class of reagents is the one containing hydrazido groups. The pK_a value of a hydrazide is usually much lower than that of an aliphatic amine (Inman and Dintzis, 1969) and the use of hydrazide compounds allows a reaction to be carried out at a moderate acid pH which is suitable for protein modifications. The product of a hydrazide with an aldehyde, a hydrazone, is usually stable and does not require reduction (O'Shannessy, et al., 1987) because of the lower basicity of the nitrogen which is caused by the attractive effect from the high electronegative atom adjacent to it.

2.4.3.2 Condensation of Periodate Oxidized Glycols

Because aldehyde groups are produced as a result of periodate oxidation on carbohydrate moieties of glycoproteins, it is of interest to review the mechanism of the action of periodate ion on saccharides, and the subsequent condensation with amino or hydrazido compounds. Referring to the previously discussed proposed mechanism of periodate oxidation of glycols whereby a cyclic ester was formed between IO_4^- and the glycol (Fig. 2.3), it is reasonable to suppose that, throughout the reaction, the parent structure was retained and that the condensation occurred through the pair of carbon atoms which formed the original α-glycol group. A hemialdal linkage of this kind (Fig. 2.7, 1) was proposed to explain the absence, as deduced from spectrophotometric data, of carbonyl groups in periodate-oxidised methyl α-D-glucopyranoside (Barry and Mitchell, 1953b). This was also supported by the finding that only one molecule of the reagent had condensed with each potential dialdehyde group and accordingly a cyclic structure was proposed for the condensation products (Fig. 2.7, 2 and 3). The reagents, p-aminobenzoic acid, p-

nitrophenylhydrazine, cyclohexylamine, thiosemicarbazide, isonicotinhydrazide, have been reported to condense with dialdehyde of periodate-oxidized starch, trehalose and raffinose (Barry, et al., 1953b; 1954a; Kitsuta, 1953). Another example in this respect is that resulting from the addition of the periodate oxidation product of adenosine 5'-phosphate and methylamine. In this case a cyclic morpholine derivative is formed (Khym, 1963). It was reported that the stability of the addition complexes was dependent upon the pH of the solution (Khym, 1963).

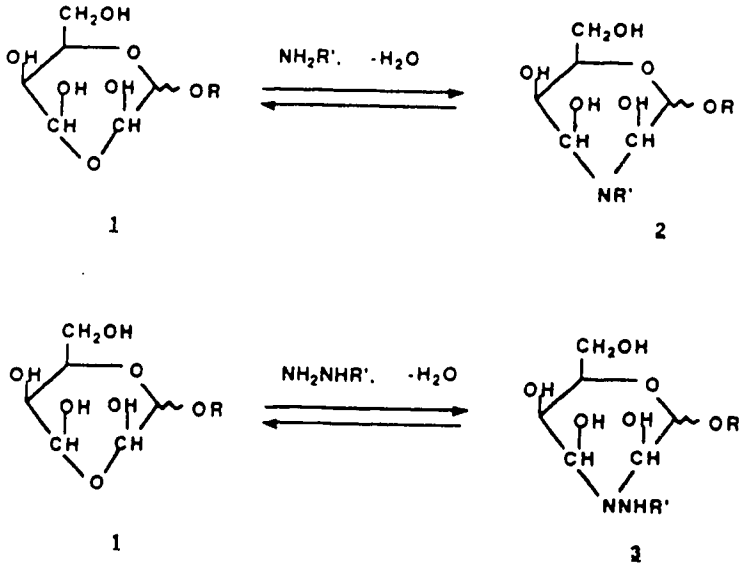


Fig. 2.7 Proposed condensation scheme of periodate-oxidized saccharide with amino and hydrazide compounds

The reported manipulation of oligosaccharide moieties of IgG molecules with amino compounds includes the spin labeling of polyclonal IgG for the study of immunoglobulin structure (Willan, et al., 1977; Nezlin, at al., 1982); labeling with enzymes and amino acids (Murayama, et al., 1978); and the attachment of a chelating agent, an amino derivative of DTPA (Rodwell, et al., 1986). Hydrazide compounds used in the condensation reaction include biotin hydrazide, fluorescent hydrazides (Weber, et al., 1975; O'Shannessy, et al., 1984, 1985), enzymes linked with hydrazide groups (O'Shannessy and Quarles, 1985), and liposomes containing

hydrazide functionalities (Chua et al., 1984). In addition, agarose hydrazide was utilized in the preparation of immunoaffinity supports by this approach (Hoffman, et al., 1988).

2.4.4 The *in vivo* Role of Carbohydrate Moieties on Immunoglobulins

Reports on the influence of terminal sialic acids on survival of IgG molecules in circulation are contradictory, particularly in light of published papers on the fate of other plasma glycoproteins such as orosomucoid and fetuin. The studies on the catabolism of IgG subunits show that the submolecular structure responsible for persistence in the circulation and catabolism of Ig is located on the Fc fragment (Spiegelberg, et al., 1965). Results of these investigations also showed that removal of sialic acid from intact IgG and papain Fc fragment did not affect their elimination and catabolism. Another experiment conducted by Mattes (1987) showed that there was no observed increase in liver uptake after neuraminidase treatment of 5 subclasses of IgG molecules, although the author did not exclude the possibility of the resistance of these to neuraminidase treatment. This is probably because liver uptake and catabolism of IgG is governed by a mechanism different than that for most other plasma glycoproteins.

Two chemical treatments of antibodies, addition of simple aliphatic compounds by reductive alkylation, and carbodiimide-promoted amide formation, have been evaluated for their influence on the clearance from the circulation (Winkelhake, 1977). These investigations showed that stoichiometric additions did not alter antibody survival time in circulation or antigen-binding capabilities. However, modifications involving excess reagent (carbodiimide or sodium borohydride) resulted in significant changes in both clearance and antibody activities. In one study where glycoproteins were treated by periodate oxidation or enzymatic modification for terminal sialic acid it was shown that a 7-carbon sialic acid analogue (after elimination of C-8 and C-9 by oxidation-reduction) mimics sialic acid with respect to hydrolysis by neuraminidase and in regulating survival of the glycoproteins in circulation. Upon injection into rats, radiolabeled preparations of

ceruloplasmin and orosomucoid treated with above methods exhibited a normal half-life in the plasma.

The carbohydrate-deficient antibodies produced by hybridoma cells with tunicamycin were tested for their biological behaviour (Nose and Wigzell, 1983). It was found that these antibodies behave in an identical manner to normal antibodies with regard to fine antigen-binding reactivity. But it was also found that antigen-antibody complexes produced from such carbohydrate-deficient antibodies were not eliminated rapidly from the circulation. Tao and Morrison (1989), in their studies using aglycosylated chimeric mouse-human IgG, found that the serum half-life of aglycosylated IgG1-Gln (Asn-297 is changed to Gln) in mice remained the same as that of wild-type IgG1. The results suggested that the asialoglycoprotein receptor may not play a role in clearing human IgG1 in mice. Clearly, more evidence is needed in this area to better understand the underlying mechanisms of immunoglobulin clearance as a result of carbohydrate moiety manipulations.

2.5. Radioimmunoimaging and Radioimmunotherapy with Monoclonal Antibodies

2.5.1 Selection of Suitable Radionuclides

A number of recent reviews have summarized the considerations for choosing radionuclides in radioimmunoimaging and therapy (Brady, et al., 1987; Bhargava, et al., 1989; Wolf, et al., 1986; Otsuka, et al., 1987; Halpern, et al., 1986; Britton, et al., 1991a,b). The type of radionuclides, and the desired physical and chemical properties, half life, energy, type of radiation are of first consideration. The next consideration is the availability of the radionuclides and the chemical methods available to incorporate them on antibodies. The *in vivo* kinetics of bio-distribution of a radiolabeled antibody is also of great importance.

For a radionuclide to be useful in a diagnostic or a prognostic procedure, it is desirable that such radionuclide deliver a high-photon density, preferably between 100 KeV and 200 KeV. This is an ideal radiation energy range for imaging devices currently used to achieve high resolution. The half life of the photon emitter should

be as short as possible and also compatible with the biodistribution pattern of radiolabeled antibodies. For example, the 2.8-day half life, 173 KeV and 247 KeV of photon energies of ^{111}In should be suitable for tumor imaging 2 days after injection, with a biodistribution pattern reaching an optimal tumor-to-tissue ratio at this time. Whereas short-lived ^{123}I and ^{99m}Tc would be more suitable for radiolabeled antibody fragments which have a fast clearance rate from the circulation. A further consideration should be given to the fact that the cyclotron-produced ^{123}I is not as readily available as the generator-produced ^{99m}Tc. Table 2.2 gives some of the radionuclides used in radioimmunoimaging studies (Brady, et al., 1986; Otsuka, et al., 1987; Wolf, et al., 1986).

Table 2.2 Physical characteristics of selected radionuclides used in labeling monoclonal antibodies for radioimmunoimaging[a]

Isotope	$T_{1/2}$	Decay mode	Gamma energy (Kev)
^{131}I	8.05 d	$\beta^-(\tau)$	284(5%) 364(82%) 637(7%)
^{123}I	13.10 h	EC[b]	159(83%)
^{111}In	2.83 d	EC	171(88%) 247(94%)
^{99m}Tc	6.02 h	IT[c]	140(89%)
^{67}Ga	3.26 d	EC	184(24%) 296(19%)
^{68}Ga	1.20 h	β^+	511(178%)
^{125}I	60.0 d	EC	27(138%)[d]

a: Table generated from Wolf, et al. (1986); Otsuka, et al. (1987)

b: Electron capture

c: Internal conversion

d: For biodistribution study

For therapeutic purposes, it is desirable to deposit as much energy as possible at target sites, while causing minimal toxicity to non-targeted tissues. Particle emitters (α or β) with a high energy and a short range should be used. Some useful radionuclides used in radioimmunotherapy are listed in Table 2.3.

As can be seen from Table 2.3, ^{131}I is less effective than ^{90}Y in terms of energy delivery. The half life of 8 days is somewhat longer than that needed and existence of high gamma radiation (364 KeV) in addition to possible dehalogenation of radioiodinated antibodies *in vivo* may contribute a significant amount of radiation exposure to the thyroid and other non-target organs. The pure β^- emission of ^{90}Y

is thus more suitable for radioimmunotherapy purposes. Despite these stated drawbacks, ^{131}I still provides a popular alternative in radioimmunotherapy because of the ease of labeling, an inexpensive cost, and ready availability. The final choice of a radionuclide is predicated by the availability of a reliable method of labeling, the production of a stable product *in vivo*, and a reduction of non-target uptake. Table 2.4 summarize the advantages and disadvantages associated with various radionuclides used for diagostic and therapeutic purpose.

Table 2.3 Radionuclides useful for radioimmunotherapy[a]

Isotope	$T_{1/2}$	Decay mode	energy (Mev)[b]	g-rad/μCi-hr
^{211}At	7.2 h	α	5.9(41%)	5.21
^{212}Bi	60.5 min	α	6.1(25%)	3.25
^{67}Cu	61.8 d	β^-	0.14(100%)	0.30
^{131}I[c]	8.0 d	β^-	0.18(99.9%)	0.38
^{32}P	14.3 d	β^-	0.70(100%)	1.48
^{188}Re	17 h	β^-	0.76(100%)	1.63
^{186}Re	3.8 d	β^-	1.07_{max}	
^{47}Sc	3.4 d	β^-	0.16(100%)	0.35
^{90}Y	64.1 h	β^-	0.93(99.9%)	1.99
^{153}Sm[c]	46.8 h	β^-	0.64_{max}	
		γ	0.103(28%)	

a: Table generated from Brady, et al. (1986)

b: Average energy unless specified.

c May be used for both imaging and therapy

Table 2.4 Comparison of some commonly used radionuclides for radioimmunoimaging and radioimmunotherapy[a]

Isotopes	Use	Major Advantages	Major Disadvantages
^{99m}Tc	Imaging	Physical properties	Labeling Short $T_{1/2}$
^{111}In	Imaging	Physical properties Labeling	Liver and spleen uptake
^{123}I	Imaging	Physical properties Labeling	Availability In vivo instability
^{131}I	Imaging	Availability Labeling	Physical properties In vivo instability
^{131}I	Therapy	Availability Labeling	In vivo instability
^{90}Y	Therapy	Physical properties	Bone uptake
^{186}Re	Therapy	Physical properties	Availability Labeling
^{188}Re	Therapy	physical properties	Availability Labeling
^{67}Cu	Therapy	Physical properties Labeling	Availability
^{211}At	Therapy	Physical properties Labeling	Availability In vivo instability

2.5.2 Radioiodination

Radioiodine labeling of antibodies and proteins has been used long before other radiolabeling methods in radioimaging and biodistribution studies. An excellent review of radioiodination methods was recently published by Bhargava, et al. (1989). A number of other reviews and reports also summarize recent developments in radioiodination (Otsuka, et al., 1987; Brady, et al., 1987; Divgi, et al., 1989; Hnatowich, et al., 1990b). Two methods commonly used in the radioiodination of proteins are the Chloramine-T (Hunter and Greenwood, 1962) and the Iodogen approaches (Pierce, Fraker and Speck, 1978). These are simple and efficient methods. To terminate the reaction in the Chloramine-T method a reducing agent, NaS_2O_5, is added. In the case of the Iodogen method the reaction is simply stopped by removing the aqueous reaction mixture to another vial because of the insolubility of the Iodogen in aqueous media. It has also been reported that the Iodogen method was less damaging to the biological properties of antibodies and was preferable to use (Salacinski, et al., 1981; Wong, et al., 1988; Turner, et al., 1988). Detailed studies on conditions of radioiodination with the Iodogen method have been reported by Saha, et al. (1989).

Other direct iodination methods involving enzymatic and electrolytic methods and indirect labeling using Bolton-Hunter reagent have not been widely used in the radioiodination of proteins. The electrolytic method results in higher aggregates and a lower specific activity. The enzymatic method needs an additional separation step since the enzymes used may be radioiodinated as well in the reaction mixture.

Direct radioiodination methods involve the formation of a cationic species of iodine, and the labeling occurs mainly on the phenolic ring of tyrosine residues of proteins (electrophilic attack). Indirect radioiodination with such reagents as the Bolton and Hunter reagent entails the conjugation of the reagent's radioiodinated phenyl ring to proteins *via* the ϵ-amino lysine residue on the protein. The reagent has an active ester responsible for this reaction.

A common problem with radioiodinated proteins is *in vivo* instability, presumably due to dehalogenation, mainly in the liver by deiodinases that can not

distinguish between tyrosine and thyroxine. Dehalogenation occurring at the tumor site was also reported (Khaw, et al., 1986, Zalusky, et al., 1985, Turner, et al., 1988). Khaw, et al. (1986) utilized a dual-labeled MAb (^{125}I and ^{111}In) with the idea that if one of the radiolabels alters biological distribution of the molecule as a whole, then both radionuclides should behave in a similar manner, provided both are still associated with the antibody. Their data demonstrated that there was a progressive decrease of iodine radioactivity and a rise in the ratio of In-to-iodine radioactivity in the tumor. One of the explanations given speculated that dehalogenation occurred there.

Two improved procedures have been reported (Zalutsky, et al., 1987, 1988; Wilbur, et al., 1986, 1989; Knawli, et al., 1989) in an effort to minimize the similarity of dehalogenation with thyroxine, and hopefully to develop a more stable, nonphenolic ring radioiodination approach. The procedures described involved the use of an organometallic intermediate, N-succinimidyl m (or p)-(tri-n-butylstannyl)benzoate 4 (Fig. 2.8). Radioiodine labeled N-succinimidyl para or meta-iodobenzoate 5 (Fig. 2.8) was first prepared. A radioiodinated succinimide ester was then covalently conjugated to available amino groups on proteins. A largely reduced thyroid uptake, rapid clearance of radioactivity from normal tissues and increased tumor uptake was observed in animal studies upon injection of a radioiodinated protein labeled by this procedure. This procedure has recently been tested in a small series of patients (cited from Hnatowich, 1990b). The complete absence of radioactivity in thyroid, stomach, and gut suggested that stability towards dehalogenation occurred in patients as well. A comparative study with a conventional radioiodination method, indicated that tumor accumulation was identical for antibodies labeled by both methods.

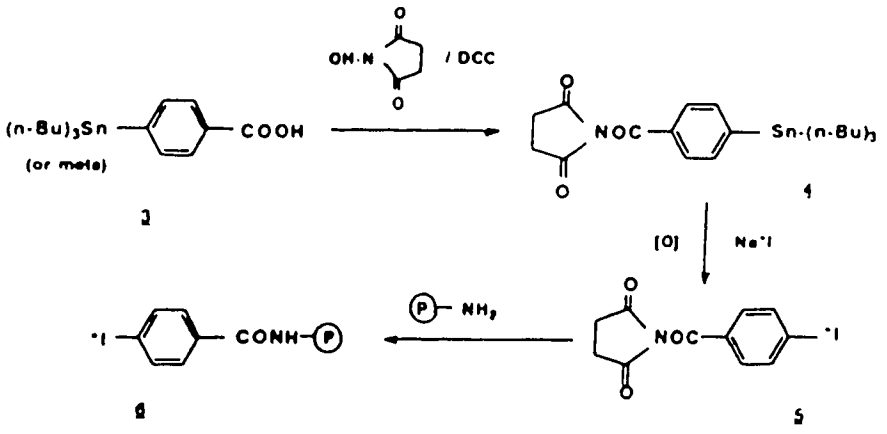


Fig. 2.8 Synthesis of N-succinimidyl-m(or p)(tri-n-butylstannyl)-benzoate and radioiodination of proteins

2.5.3 Direct Labeling of Antibodies with ^{99m}Tc and ^{111}In

Because of the ideal physical properties of ^{99m}Tc for radioimaging using conventional and single photon emission computed tomography (SPECT), the use of this radionuclide has been explored for the potential application in the labeling of antibodies. One of the major areas of interest in ^{99m}Tc labeling of antibodies is the direct labeling without precoupling with a chelating agent. Recent reviews for direct labeling of ^{99m}Tc to antibodies and their clinical prospects have been given by Eckelman, et al. (1986); Saccavini, et al. (1986); and Hnatowich (1990b).

Direct labeling methods with ^{99m}Tc or ^{111}In were generally considered to be unstable *in vivo*. Paik, et al., (1985a) studied the stability of the direct labeling of ^{99m}Tc of the intact antibody, F(ab')$_2$ and Fab fragment in the presence of free DTPA. Two binding sites for F(ab')$_2$ and intact IgG were suggested. One with a

high capacity, low affinity site and another having a low capacity, high affinity site which can compete with the presence of free DTPA. Because of the lack of a high affinity site on the Fab fragment it was suggested that the high affinity site may be related to the presence of sulfhydryl groups on antibodies.

A direct labeling method with ^{99m}Tc called 'pre-tinning' for a number of monoclonal antibodies and antibody fragments was reported by Rhodes, et al. (1982, 1986). In this approach antibodies are first incubated with stannous ions (0.005 M Sn^{++}) in phthalate and tartrate solution at a pH 5.6 for 21 hours and then pertechnetate in normal saline solution is added. The reduced ^{99m}Tc is chelated by sulfhydryl groups obtained from the 'pre-tinning' step. The assumed sulfhydryl chelation with ^{99m}Tc is derived from the conversion of $F(ab')_2$ to its monomer Fab' fragment during the labeling which splits the disulfide bonds that normally bridge the two monomers together. ^{99m}Tc-labeled antibodies and antibody fragments by this method possess high stability to transchelation by EDTA *in vitro*. The method requires purification by passing the reaction mixture through a filter column (Filtech filter) specially designed to remove stannous ions, colloid, residual pertechnetate, and reversibly bound technetium. Purified ^{99m}Tc-labeled antibodies and antibody fragments were shown to be capable of binding to human tumor xenografts in nude mice. The method was also used in a commercial kit.

Following similar principles, different reducing agents were tested in an attempt to replace stannous chloride, and in the hope of developing a simpler procedure and a better radiochemical yield for kit preparation. Two such reducing agents, 2-mercaptoethanol (Schwarz, et al., 1987; Baum, et al., 1989; Mather, et al., 1990) and dithiothreitol (Pak, et al., 1987) have been reported. Preincubation time was reduced to only 10 minutes at room temperature. In addition, a weak complex of reduced ^{99m}Tc with phosphonate or pyrophosphate, and glutarate have been used instead of sodium pertechnetate to reduce non-specific binding of ^{99m}Tc to low affinity sites other than sulfhydryl groups on antibodies. High radiochemical yields made purification of postlabeling unnecessary. The absence of thyroid uptake in clinical trials proved the stability of the ^{99m}Tc-labeled antibodies towards *in vivo*

formation of pertechnetate. Low liver uptake suggested the absence of radiocolloid. Many studies in patients have shown the potential utility of these agent for radioimmunoscintigraphy (Baum, et al., 1989).

Not much work has been reported on the direct labeling of ^{111}In to antibodies. Extended studies on the (Sn)citrate chemical species used for radiolabeling with ^{99m}Tc has led to the finding that indium could be labeled in a similar way (Wong, 1987). The initial reaction of sodium citrate with $SnCl_2$, or SnF_2 in an aqueous medium was followed by the formation of a bimetallic In-(Sn)citrate complex species at pH 7.4 after the reaction of (Sn)citrate with $^{111}InCl_3$ at 120°C for 15 minutes. The In-(Sn)citrate complex was then covalently coupled to proteins at 37°C. The reported labeling efficiency was greater than 98% and the biochemical activity of the radiolabeled proteins was stable in excess of 3 months when stored at 2-8°C. The labeling mechanism is not well understood. The authors also emphasized that the labeling procedures must be strictly followed for optimal binding of radionuclide to protein molecules.

2.5.4 Radiolabeling *via* Bifunctional Chelating Agents

The attractive physical properties of metallic radionuclides suitable for radiodetection and radiotherapy have stimulated the interest in the development of methods suitable for the radiolabeling of proteins. Since most proteins lack the ability to chelate radiocations directly, an indirect method involving the use of bifunctional chelating agents, having both abilities to chelate desired metals and to conjugate to proteins, has been developed. The first report of attachment of metal binding groups to proteins was by Gelewitz, et al. (1954). These authors reported the coupling of azo-phenanthroline and azo-oxine to albumin. But the pioneering work in obtaining stable conjugates for *in vivo* use was not explored until Sundberg, et al. (1974a,b) reported the first useful bifunctional chelate for the labeling of proteins. The compound used was azophenyl derivative of ethylenediaminetetraacetic acid (EDTA). Following this initial report, a number of others exploring new methods of chelating metals and coupling to proteins were

described for possible clinical applications. Stability of metal chelates tested *in vivo* was generally considered as one of the critical features. Although a large number of chelates have been synthesized, only a few basic chelates have been used for antibody labeling with the most widely used radionuclides, ^{111}In and ^{99m}Tc. Some recent reviews have been reported on this matter (Meares, 1986; Eckelman, et al., 1986; Otsuka, et al., 1987; Bhargava, et al., 1989; Hnatowich, 1990b).

2.5.4.1 ^{111}In Labeling

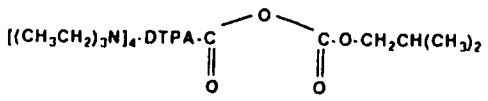
Derivatives of EDTA and diethylenetriaminepentaacetic acid (DTPA) are among the major chelating agents used for ^{111}In labeling of antibodies. Because of their stronger chelating properties with indium, DTPA derivatives have been used more extensively. The structures of DTPA and EDTA and some of their derivatives used in the conjugation to antibodies are illustrated in Fig. 2.9 and Fig. 2.10.

^{111}In labeling is usually achieved after chelating agents are covalently coupled to proteins. General labeling procedures involve a starting step in which $^{111}InCl_3$ is chelated to a weak complex such as acetate or citrate at pH 5.5 to pH 6.0. Formation of In-acetate or In-citrate prevents the precipitation of $In(OH)_3$ (colloid) which otherwise would occur at this pH range. ^{111}In labeling of proteins is then achieved by transchelation of ^{111}In from acetate or citrate to a higher affinify chelating agent already coupled to the protein. This method has been very successful in the Indium labeling of proteins with high radiochemical yields (>98%), rapid reaction at room temperature (about 15 min), and generally no radiocolloid formation.

New coupling methods have been explored while searching for simple methods of synthesis of chelate derivatives. In addition, improved conjugation procedures to minimize the damage to antibody immunoreactivity, and reduce aggregate or oligomer formation have attracted prime interest. Some of these conjugation approaches are summarized as follows.

1. 1-(p-benzenediazonium)-EDTA (Fig. 2.10, B) was among the first ones used to attach a metal ion to proteins with relative stability *in vivo* (Sundberg, et al.,

A: Mixed Isobutylcarboxycarbonic anhydride of DTPA

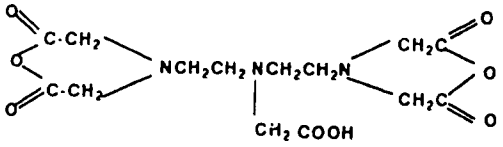


(Krejcarek, etal., 1977)

(Paik, etal., 1983a)

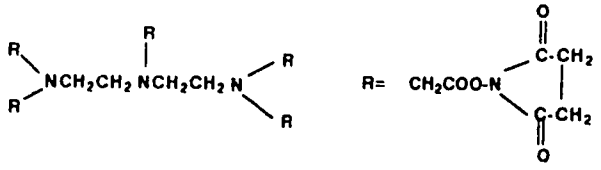
B: Cyclic dianhydride of DTPA

(Hnatowich, et al., 1982)



C: N-Hydroxysuccinimide ester of DTPA

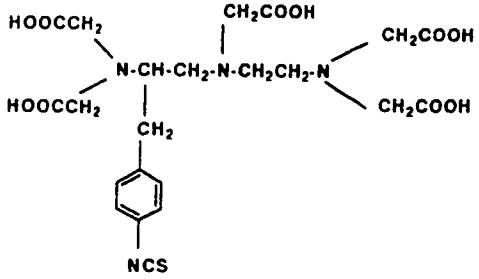
(Najafi, et al., 1984)



D: 1-(p-Isothiocyanatobenzyl)-DTPA

(Brechbiel, et al., 1986)

(Westerberg, et al., 1989)



E: Benzylisothiocyanate derivative of DTPA

(Sumerdon, et al., 1990)

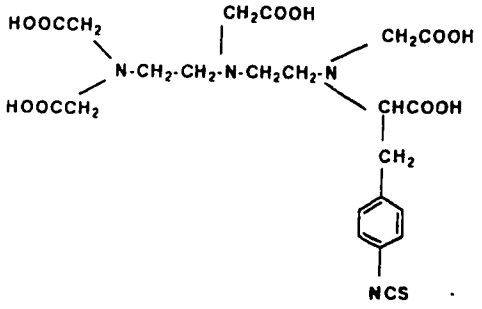
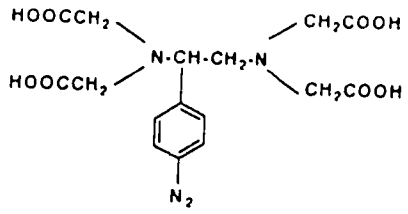
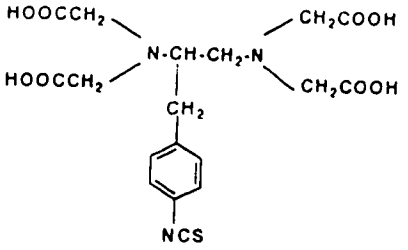


Fig. 2.9 Derivatives of DTPA

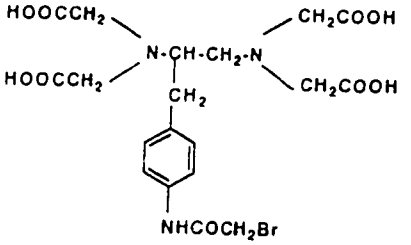
A 1-(p-benzenediazonium)-EDTA (Sundberg et al., 1974a,b)



B: 1-(p-isothiocyanatobenzyl)-EDTA (Meares et al., 1984)



C: 1-(p-bromoacetamidobenzyl)-EDTA (Meares et al., 1984)



D: Benzylisothiocyanate derivative of EDTA (Sumerdon, et al., 1990)

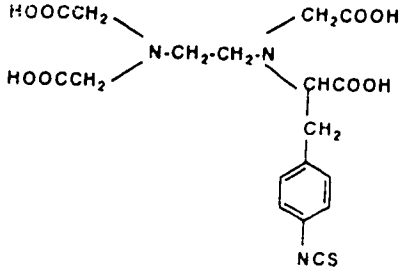


Fig. 2.10 Derivatives of EDTA

1974a,b; Meares, et al., 1976; Leung, et al., 1978). In general, diazonium coupling occurs mainly at tyrosine, histidine and lysine residues of proteins (Leung, et al., 1978). *In vitro* and *in vivo* studies on the transchelation of metal ion from the chelating group to transferrin and other serum proteins demonstrated kinetic inertness of the chelate (Meares, et al., 1976). However, it was later observed that there was rapid plasma disappearance and excretion of the radiolabeled protein when compared to the native one in cancer patients, thus indicating that the albumin labeled by this method had indeed been altered by the coupling procedure.

2. DTPA has become a more attractive agent for ^{111}In labeling because its complex with ^{111}In is quite inert to ligand exchange to transferrin (Yeh, et al., 1979a). There are two types of anhydride derivatives of DTPA which have been reported in the literature. One method involves the adoption of the mixed anhydride technique commonly used in the peptide synthesis (Krejcarek and Tucker, 1977; Ecklman, et al., 1986) (Fig. 2.9, A). The mixed anhydride is prepared by reaction of pentatriethylammonium DTPA with isobutylchloroformate. The resulting carboxycarbonyl mixed-anhydride of DTPA is then coupled to proteins. Optimal conditions of DTPA mixed-anhydride reaction with antibodies were investigated by Paik, et al. (1983a). Their data indicated that mixed anhydride was very unstable in the presence of trace amounts of water.

Another approach in this category developed by Hnatowich, et al. (1982) involves a cyclic anhydride of DTPA which is more stable than carboxycarbonyl mixed-anhydride derivative (Fig. 2.9, B). This method has been investigated extensively (Hnatowich, et al., 1983a, 1983b; Paik, et al., 1983) and been widely used in clinical trials because of its simplicity, and efficiency of methodology. Because of the existence of two active sites of cyclic anhydride DTPA cross-linking of antibody molecules may occur during the conjugation process due to improper handling, and this may result in high liver uptake. However, careful control of reaction conditions practically eliminates the latter problem.

3. Utilization of carbodiimide-activated carboxyl group in peptide and protein synthesis is a well known reaction. The water soluble carbodiimide was used to

activate carboxy groups of proteins. The intermediate was then reacted with a nucleophile (DeNardo, et al., 1977). This method is also adopted for use in conjugating a carboxyl group of DTPA to amino groups of antibodies (Brechbiel, et al., 1986). The effect of pH, the ratios of carbodiimide to DTPA, as well as DTPA to antibody were investigated (Eckelman and Paik, 1986). However, low conjugation yields and loss of immunoreactivity were observed when compared with the cyclic anhydride method.

N-hydroxysuccinimide active ester of DTPA is another alternative to conjugate DTPA to proteins (Najafi, et al., 1984) (Fig. 2.9, C). The pentaester was reported to be more stable than cyclic anhydride. However, the conjugation of DTPA to proteins by pentaester caused a high degree of dimer and polymer formation since all 5 carboxyl groups of the chelating agent (DTPA) were activated. This was later improved by synthesis of a monoester of DTPA (Paxton, et al., 1985), and it was reported that formation of monoester can be controlled by carefully manipulating the molar ratio of the reagent to protein.

4. Isothiocyanate and bromoacetamide derivatives of EDTA were also utilized for conjugation to antibodies (Meares, et al., 1984). Indium labeled EDTA conjugates *via* isothiocyanate and bromoacetamide derivatives to proteins were reported to have lower ^{111}In releasing rate than those of cyclic anhydride DTPA coupled ones (Yeh, et al., 1979b). For explanation of these results, it was suggested that the 4 remaining carboxylates on DTPA, after one was used in linking the chelate to the antibody, could lose chelated Indium relatively faster than where all 5 carboxylates are available (Bhargava, et al., 1989). It was also suggested that one of the causes accounting for increased liver uptake of ^{111}In-labeled antibodies might be the consequence of ^{111}In transchelation to transferrin which in turn localized in liver and other reticuloendothelial systems. Nevertheless, other factors may also have played a role in the stability of ^{111}In-chelates since EDTA type of chelates which contain only 4 carboxyl groups behaved in a rather stable way. Formation of dimers or aggregates using cyclic anhydride technique in conjugation reaction is probably also responsible for the high liver uptake (Carney, et al., 1989).

Recently a new chelating agent, 1-(p-isothiocyanatobenzyl)-DTPA (SCN-DTPA) (Brechbiel, et al., 1986) (Fig. 2.9, D) in which all five DTPA carboxylate groups are conserved for binding metal ions has been synthesized. Reduced liver radioactivity and improved tumor to organ ratio were observed in animal tests (Blend, et al., 1988; Carney, et al., 1989; Khaw, et al., 1990).

An analogue of SCN-DTPA that differs only in that the isothiocyanatobenzyl group is attached to a methylene carbon on one of the acetic acid groups rather than to a backbone ethylene carbon was also reported (Westerberg, et al., 1989) (Fig. 2.9, D). In this case all five carboxyl groups are available for chelating with ^{111}In. This chelate has also been compared with cyclic anhydride-DTPA and SCN-EDTA, and the results of such comparison showed lower liver uptakes for both SCN-EDTA and SCN-DTPA conjugates while higher liver uptake were observed for cyclic anhydride-DTPA conjugate in animal tests. (Carney, et al., 1989; Sumerdon, et al., 1990).

5. Modification of carbohydrate moieties of antibodies was one of the areas further explored. The oligosaccharide moieties of antibodies are located on the constant Fc region which is not related to the antigen-antibody binding process. A survey in this area was given in section 2.4. Glycyltyrosyllysyl-DTPA (GYK-DTPA) and p-aminoaniline-DTPA were among the first compounds used for the labeling of ^{111}In on the carbohydrate moieties of antibodies for radioimmunoimaging (Rodwell, et al., 1986). A summary on this subject is given by several review articles (O'Shannessy, et al., 1987; Zuckier, et al., 1989).

Preservation of immunoreactivity of antibodies after manipulation with this method has also been reported by a number of researchers (Rodwell, et al., 1986; Brown, et al., 1986; Alvarez, et al., 1988; Chua, et al., 1984). The general strategies used in the conjugation (Murayama et al., 1978; Blair et al., 1983; Rodwell et al., 1986; O'Shannessy et al., 1987; Alvarez et al., 1988; Perala et al., 1990) are: a) carbohydrate moieties of antibodies are oxidized by incubation of an antibody solution with 10-30 mM of $NaIO_4$ for 30 to 60 minutes b) the oxidized antibodies are freed from excess of $NaIO_4$ by a gel filtration column c) incubation with an

excess of metal chelates, such as 500 to 1000 fold in order to minimize potential intermolecular or intramolecular condensation (O'Shannessy et al., 1987) with oxidized antibodies, for 1 hour. This is followed by addition of $NaBH_3CN$ to a final concentration of 10 mM overnight and removal of small molecules by dialysis or a gel filtration column. The flow diagram Fig. 2.11 outlines the procedure of periodate oxidation of antibodies and subsequent conjugation to amino or hydrazine/hydrazide compounds.

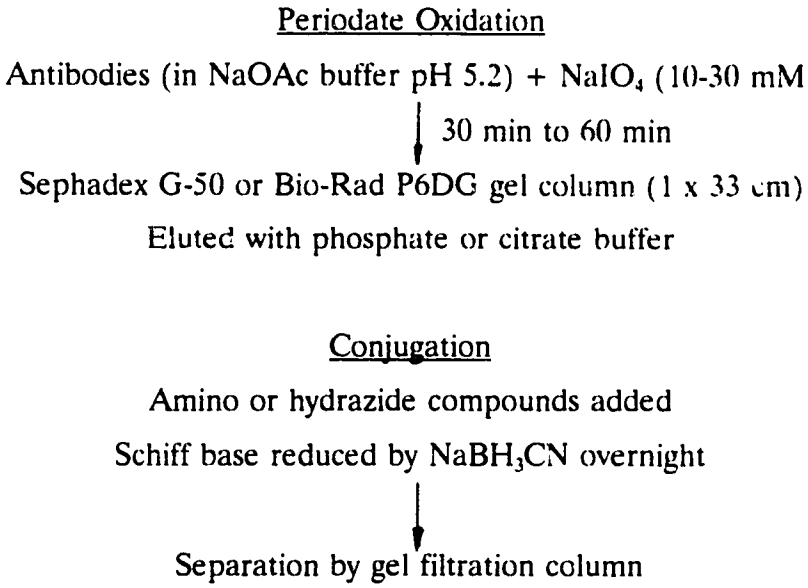


Fig. 2.11 Flow diagram of the modification of oligosaccharide moieties of antibodies

2.5.4.2 Indirect Labeling of Antibodies with Technetium-^{99m}

Since DTPA was reported to form stable chelates with many metal ions it became the first choice for investigating the indirect chelation of ^{99m}Tc to proteins (Lanteigne, et al., 1984; Child, et al., 1985). It was soon realized that DTPA could not provide a chelate with ^{99m}Tc which was stable enough to prevent non-specific binding of reduced ^{99m}Tc to low affinity sites on antibodies (Hnatowich, 1990b). Franz, et al. (1987) reported ^{99m}Tc-labeling of antibodies using a cyclam (macrocyclic amine ligand)-based chelating agents. Technetium-^{99m} labeling was conducted prior to conjugation of the chelate to the antibodies because of low

labeling yields (4%).

The diamide dithiolate ligand system (N_2S_2) forms stable, well defined tetradentate complexes with Tc(V). This series of ligands has also been investigated for its use in labeling of antibodies with ^{99m}Tc (Fritzberg, et al., 1988). Antibodies and their fragments were labeled by conjugation of prelabeled ^{99m}Tc-4,5-bis(thioacetamido)pentanoate active ester to protein amino groups. Since difficulties existed in eliminating non-specific binding by this procedure, the chelate was first radiolabeled and then conjugated to antibodies. Postlabeling purification was needed as well. Thus, alternate chelating agents are required if indirect ^{99m}Tc conjugation to proteins is to become a practical reality.

2.5.4.3 ^{90}Y Labeling

Recently some success in ^{90}Y labeling of antibodies has been obtained. Yttrium-^{90} is considered to be one of the optimal radionuclides for use in radioimmunotherapy because of suitable half-life (2.6 days), pure and high energy ß$^-$ emission, and ready availability from a generator. Cyclic anhydride DTPA was investigated in the radiolabeling of antibodies with ^{90}Y (Hnatowich, et al., 1988). A stable ^{90}Y-labeled antibody conjugate was obtained with a dissociation rate of 8-9% per day. It was suggested that ^{90}Y-labeled MAb may be suitable for radioimmunotherapy (Lee, et al., 1990).

Since radiotherapy involves a process to kill the target cells, the *in vivo* biodistribution of radiolabeled conjugates becomes very critical. In the case of radioimaging, optimal imaging can be obtained by selecting a time when an optimal target-to-background ratio is reached. These methods which provide useful information in radioimaging may not necessarily be suitable for radiotherapy. Two methods for the ^{90}Y labeling of antibodies, either by cyclic anhydride DTPA (c-DTPA) conjugated to amino side chains of the antibody or p-NH_2-Bz-DTPA conjugated to the carbohydrate moiety of the antibodies were compared for possible use in radioimmunotherapy (Lee, et al., 1990). Animal test results showed that the tumor size was unchanged from base line, at a dose of 200 μCi/25 g mouse for a

preparation incorporating cyclic anhydride DTPA. In contrast a maximum tumor volume reduction of 87% was observed for animals receiving an antibody preparation labeled via p-NH_2-Bz-DTPA in the same dosage. The results were very encouraging. These authors also suggested that toxicity could be reduced by improvements in bifunctional chelating techniques.

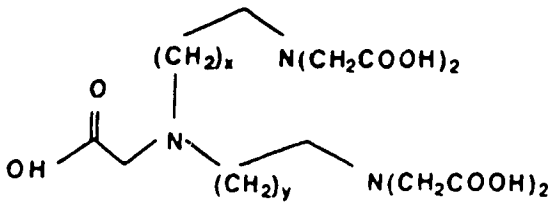
2.5.5 Development of New Chelating Techniques

Higher background radioactivity in the liver, spleen, kidney, bone, and a low uptake by tumors are the greatest difficulties encountered by the use of the radiolabeled MAb technique. Beside the improvement of specificity of MAbs to target tumors and the reduction of the effect of murine MAb on human imaging, improving the methods by which antibodies are radiolabeled remains a most important objective. There are several reports indicating that antibodies are affected differently by various labeling procedures (Otsuka, et al., 1987). Different approaches may be needed for the labeling of individual antibodies (Eckelman, et al., 1986). The procedure adopted should be simple and easy to handle. Most importantly, such methods should induce only minimum damage to the immunoreactivity of antibodies.

A number of new chelating agents have been reported (Richard, et al., 1990). One of a series of compounds, ethylenepropylene-triaminopentaacetic acid (N_2N_3) (Fig. 2.12) has been conjugated to antibodies by a cyclic anhydride method and labeled with ^{111}In. Enhanced tumor-to-blood ratios (9 vs 6.5) and decreased liver uptake (4 vs 12%) were observed when comparison was made to cyclic DTPA. Because the cyclic anhydride method was used in the conjugation reaction for both chelators, the difference observed in liver uptakes and blood levels might be the result in the difference between the stabilities of both chelates. However, there is no direct evidence to support such a claim.

Another series of chelating agents used for tumor targeting are macrocyclic chelators. These type of agents have been reported to be less sensitive to acid catalysed dissociation pathways and more kinetically inert at lower pH as may be

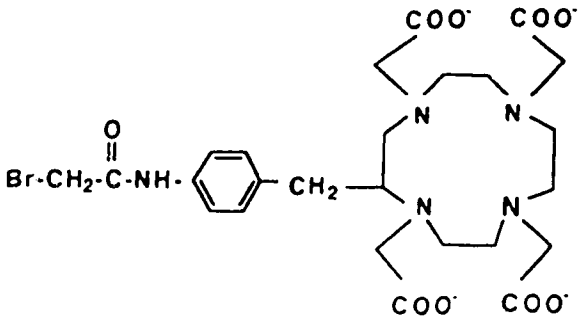
encountered in the stomach or liver tissues (Parker 1990). Tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA) (Fig. 2.13) was recently used in the ^{90}Y labeling (Deshpande and Meares, et al., 1990). It was shown during serum stability studies that transchelation of ^{90}Y from DOTA to serum proteins is essentially undetectable. This was similar to previous findings for Indium chelation with DOTA. Ring size of this type of chelating agents can be adjusted to fit the desired nuclides. Inserting a spacer between the antibody and chelating agent was reported to be necessary in order to obtain a stable labeling (McCall, et al., 1990).



N_2N_3 : X = 2, Y = 3.

(Richard, et al., 1990)

Fig. 2.12 Structure of a new series of chelating agents



(Deshpande, et al., 1990)

Fig. 2.13 Structure of a derivative of the new chelating agent, DOTA

2.5.6 Other Approaches to Reduce the Radioactivity in Normal Tissues

A major difference between various chelating methods is the chemical bond by which such chelates are attached to antibodies. A new approach introducing a

metabolizable linkage between metal chelates and antibodies may lead to a decrease in circulating non-target background activity due to enzymatic cleavage of the chelates which would then be rapidly excreted by the kidneys (Haseman, et al., 1986). An enhanced target to non-target ratio may occur if cleavage on tumor site is slower than that in the liver and other non-target organs. A new chelating agent, a derivative of hydroxyethyl-EDTA was synthesized with two ester linkages susceptible to enzymatic cleavage by esterases found in the liver and other organs. Biodistribution in mice bearing B-cell lymphomas in subcutaneous tissues of flank showed a decreased blood background activity and an increased tumor-to-blood ratio at the cost of a decrease in absolute tumor uptake of chelates, particularly when compared with a non cleavable linkage (p-(bromoacetamido)benzyl-EDTA). Similar results of improved tumor-to-background ratio have been reported by Quadri, et al. (1990) in which a linkage of ethylene glycol bis(succimidyl succinate)(EGS) was introduced. Another recent report described a reduction of liver uptake *in vivo* by introducing a spacer, poly-l-lysine that may be easily cleaved *in vivo*. (Wang, et al., 1990).

Among four different linkages, disulfide, diester, thioether, and hydrocarbon, the former two linkages are reported to be labile (Paik, et al., 1989). Antibody conjugates with thioether and hydrocarbon linkages showed biodistribution patterns similar to these of a peptide-linked conjugate. While disulfide and diester linked conjugates cleared from blood much faster than these of peptide linkage, an increased target-to-blood ratios of 15 and 5, and a target-to-liver ratios of 3 and 4 respectively were obtained. Another report using four linkages between benzyl-EDTA and antibodies (Deshpande, et al., 1989) indicated that at 72 hours after injection a 2.2% and 7.6% liver uptake was observed for disulfide and thiourea linkage respectively. In addition, liver uptakes of 13.4% and 20% for thioether and peptide linkages respectively were obtained at the same time point. Fig. 2.14 illustrates some of the linkages tested between chelates and antibodies.

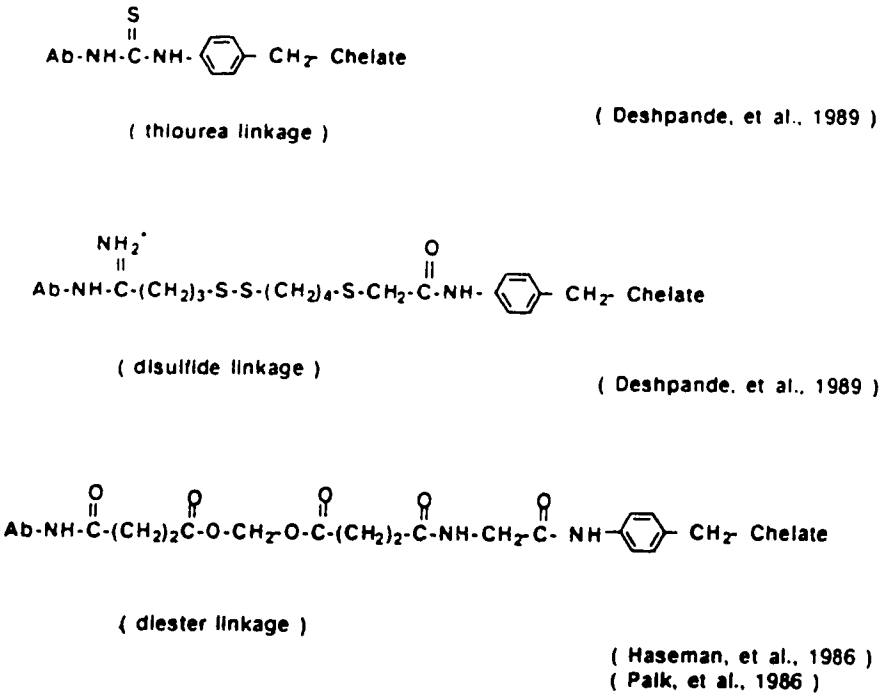


Fig. 2.14 Different linkages tested between chelates and antibodies

Several other approaches have been reported in attempts to reduce the background uptakes commonly observed with antibodies labeled with Indium and other metal conjugates. The radioimaging specificity was increased by administration of unlabeled antibodies prior to, or in conjunction with, the radiolabeled antibody conjugates (Murray, et al., 1985) One of the reasons given for better imaging results may be due to rapid saturation of non-target antigen by cold antibodies. In addition the large amount of antibodies injected may increase the uptake in tumor sites (Brady, et al., 1987).

Fab and $F(ab')_2$ fragments of antibodies are known to be cleared rapidly from the circulation. In case of tumor targeting, the molecular weight of antibody fragments has been shown to be a major determinant of capillary permeability, and consequently the ability of antibodies to reach interstitial space (Zuckier, et al.,

1989). On the other hand a more rapid excretion of antibody fragments may affect the absolute tumor uptake of the probe. Radiolabeled Fc, Fab and F(ab'$_2$) fragments were extensively evaluated for applications in radioscintigraphy. It was observed in animal tests that ^{131}I-labeled F(ab'$_2$) fragments were more efficient and less toxic than radioiodinated intact antibody in radioimmunotherapy (Buchegger, et al., 1990).

Because a very small amount of radioactivity is actually accumulated by tumors following iv injection, regional delivery of radiolabeled antibody conjugates to interstitial (lymphatic) and ip tumors has been reported. To solve some of the problem encountered in this methodology, that is to increase the rate of systemic clearance of the antibody without accelerating the rate of decline in antibody levels in ip tumors, a second antibody, unlabeled goat polyclonal anti-mouse antibody was injected (Wahl, et al., 1987). This approach resulted in the formation of immune complexes of the second antibody with antigen which was then cleared by the reticuloendothelial system.

Another recent development is reversible radiolabeling of antibodies for tumor localization. Reardan, et al., (1985) prepared monoclonal antibodies against EDTA-Indium chelates. These antibodies introduce a new control over biological distribution of chelated radionuclides and consequently alter the uptake of tumor and non-tumor organs. The processes could be explained as follows. First, a rapidly excreted, radiochelate is complexed with anti-chelate antibody (not covalently). The complex remains in the circulation for an extended period of time after injection and it may localize on tumor targets during that period. The second step is chasing with an excess nonradioactive chelate to displace the radioactive chelate in complex. This will lead to rapid clearance from the circulation (Meares, 1986). The use of a Biotin-avidin system for increased localization in the tumor as well as a low blood background level has also been proposed (Paganelli, et al., 1991, Hnatowich, et al., 1991). These methods while appearing to be extremely elegant may be too complex for commercialization and routine use.

3. MATERIALS AND METHODS

3.1 General Methods

3.1.1 Antibody Solutions

Two types of antibodies were used in this study. Polyclonal human immunoglobulin G (hIgG), as a lyophilized, essentially salt free product was purchased from Sigma Chemical Company, USA. Whenever required, the antibody was weighed and dissolved in 0.05 M sodium acetate (NaOAc) buffer, pH 5.2.

Purified MAb 155H.7 (subclass IgG_{2b}), raised against a synthetic ß-anomer of Thomsen-Friedenreich antigen was obtained from Biomira Inc., Edmonton, Canada, in 0.1 M sodium acetate buffer solution, pH 6.0. The antibody solution was stored in a refrigerator and was not frozen.

3.1.2 Estimation of Protein Concentration

Protein concentrations were determined from the UV absorption value obtained using either a LKB PU 8700 Series UV/Vis Spectrophotometer, or a Hewlett Packard 8452A Diode Array Spectrophotometer. The samples to be tested (20-40 μL) were diluted with double distilled, deionized water, resistance > 16.7 megohm-cm (DD-H_2O, Barnstead System) to concentrations ranging from 20 to 100 μg/mL. Sample concentration was determined directly if the estimated protein concentration was in above range. A value of 14 was used as a percent extinction coefficient ($\epsilon^{1\%}$) for gamma G-immunoglobulin (Williams, et al., 1968) and 6.6 for human serum albumin (HSA) (Lee, et al., 1976).

From $A = C_p \epsilon^{1\%} t$, (3.1)

where:

A = absorbance at 280 nm (A_{280nm})

C_p = percent concentration of proteins (g/100mL)

t = cell length (1 cm)

$\epsilon^{1\%}$ = percent extinction coefficient

Substituting percent concentration, C_p, in equation (3.1) with concentration of μg/mL, two equations were obtained:

$$C_M\ (\mu g/mL) = 714.3 \times A_{280nm} \quad \text{for IgG} \qquad (3.2)$$

$$C_M\ (\mu g/mL) = 1515 \times A_{280nm} \quad \text{for HSA} \qquad (3.3)$$

Protein concentrations were calculated according to either equation (3.2) or (3.3) and corrected for dilution factors.

A standard curve was constructed by a series of dilutions of hIgG solution. A correlation coefficient of 0.99 was obtained in the concentration ranges from 10 μg/mL to 150 μg/mL. The equation deduced from the standard curve was: C_M (μg/mL) = 714.8 x A_{280nm} (3.4), which was in good agreement with the one (equation 3.2) calculated with an extinction coefficient of 14.

3.1.3 Separation of Small Molecules from Conjugated Antibodies

Sephadex G-50 gel (fine) (Pharmacia Canada Inc. Pharmacia LKB Biotechnology) and Bio-Rad P-6DG gel (Bio-Rad Laboratories (Canada) Ltd., Ontario) were used in the separation.

Sephadex G-50 gel or Bio-Rad P-6DG gel was soaked in the appropriate buffer solution overnight. The swollen gels were poured into a chromatography column and equilibrated with the elution buffer. Non-specific protein binding sites on gels were saturated by passing 100 μL (1 mg) of 1% BSA (bovine serum albumin) solution through the columns before loading of samples.

Modified antibodies were separated from small molecules, such as sodium periodate, chelating agent, etc., by passing the reaction mixture through a Sephadex G-50 column (1 x 38 cm) or a Bio-Rad P-6DG column (1 x 43 cm). The eluent was monitored by UV absorbance at 280 nm equipped with an LKB Bromma UVICORD SII Monitor.

3.1.4 Analysis of Aggregate or Oligomer formation

Sepharose CL-4B gel (Pharmacia Canada Inc., Pharmacia LKB

Biotechnology) with fractionation range of 60,000 to 20,000,000 Daltons, supplied pre-swollen, was routinely used. A Sepharose CL-4B column (1 x 43 cm) was calibrated with molecular weight standards, apoferritin 443,000, hIgG 150,000 and BSA 66,000 Daltons. The column was eluted with 0.01 M phosphate buffered saline (PBS), pH 7.3. Retention time of the protein conjugate was compared with that of the standards.

For confirmation purposes, high performance liquid chromatography (HPLC) with a TSK 3000SW size exclusion column eluted with 0.01 M PBS was utilized.

3.1.5 Enzyme-Linked Immunosorbent Assays (ELISA)

Some of the modified antibodies were evaluated for their immunoreactivity by ELISA. LS174T cell homogenates and HSA were used as sources of antigen for MAb 155H.7. ELISA assay of antibody conjugates was run along with the native antibodies from which the conjugates were prepared. Each analysis was done in triplicate. The microtiter plates were coated with 150 fold diluted cell homogenate in 0.1 M PBS, pH 7.2, or 0.5 μg of HSA in 100 μL per well. The plates were air dried at room temperature overnight. The coated plates were blocked by adding 200 μL of 0.01 M PBS (containing 1% BSA) solution to prevent non-specific binding. The plates were incubated at 37^{0}C for 3 hours. The solution was then removed just prior to the assay and the plates were washed with 0.05 % Tween 20 in 0.01 M PBS three times.

Antibody conjugates were diluted with 0.01 M PBS (containing 0.1% BSA) to a starting concentration of 10 μg/100 μL and then a series of dilutions were made. One hundred μL of samples and the same amount of 0.01 M PBS solution as blank were added to each well. The plates were covered and incubated at 37^{0}C for 1 hour and then washed with 0.05% Tween 20 three times. Five μL of goat anti-mouse IgG peroxidase conjugate (affinity isolated, TAGO) in 250 fold dilution in PBS was added to each well and incubated for another 1 hour at 37^{0}C. The plates were emptied and washed four times with 0.01 M PBS solution. The final step was the addition of 50 μL of peroxidase substrates, equal parts of reagent A, 2,2'-azino-

di[3-ethyl-benzothiazoline sulfonate] and reagent B, hydrogen peroxide (Kirkegaard & Perry Laboratories. Inc., Gaithersburg, USA) to each well. The color generated was measured for absorbance at 410 nm with a reference at 490 nm by a MR600 Microplate Reader (Dynatech Laboratories Inc. Alexandria, USA) within 30 minutes. An antibody titration curve was prepared by plotting the mean optical density at 410 nm against the antibody concentration or the antibody dilution factor.

3.1.6 Native Polyacrylamide Gel Electrophoresis (Native PAGE)

Native polyacrylamide gel electrophoresis (native PAGE) was performed using a PhastSystem™ (Pharmacia LKB Biotechnology) with a PhastSystem™ gradient gel (4-15%, pH 8.8) and native buffer strips. A conjugated antibody solution in 0.1 - 0.2 M citrate buffer, pH 6.2 was diluted with DD-H_2O to 1-2 $\mu g/\mu L$. Bromphenol Blue, as an indicator for the migration front, was added to each sample to about 0.1 %. The samples were centrifuged in an Eppendorf Centrifuge for 3 minutes. About 1 μL of supernatant of each sample was applied to the gel bed. Separation conditions were as follows: Step 1, 400 V, 10.0 mA, 2.5 W, 15°C, 10 Vh; Step 2, 400 V, 1.0 mA, 2.5 W, 15°C, 2 Vh; Step 3, 400 V, 10.0 mA, 2.5 W, 15°C, 268 Vh. The procedure was terminated when the blue indicator had migrated to the top of the gel.

After separation, samples were stained by the coomassie staining technique. Coomassie, a triphenylmethane anionic dye, preferentially forms dye complexes with proteins in the gel matrix. Development conditions were: step 1, stain 7 min, 50°C; step 2, destain 1 min, 50°C; step 3, destain, 10 min, 50°C; step 4, destain 15 min, 50°C; step 5, preserving solution, 15 min, 50°C.

3.1.7 Thin Layer Chromatography (TLC)

Instant thin layer chromatography (ITLC SG, Gelman Sciences Inc. Ann Arbor, MI, USA), cellulose chromatography sheet (without fluorescent indicator, Eastman Kodak company, Rochester, N.Y., USA), and paper chromatography (Whatman #1) were used in the analysis. The specific solvent systems used are

indicated in the appropriate sections. The general procedures were as follows:

ITLC and cellulose TLC sheets were cut into 0.8 x 10 cm sections. For paper chromatography, strips of 1 x 15 cm were used. An aliquot (5 to 10 μL) of samples was spotted on TLC strips. Ascending chromatography was carried out to 1 cm from the top of each strip. After air drying the TLC strips were cut into 1 cm pieces and put into counting tubes. Radioactivity of each tube was counted in a NaI(Tl) well-counter (Beckman 8000 Gamma Counter).

3.1.8 Trichloroacetic Acid (TCA) Precipitation

A small aliquot of purified radioiodinated antibody conjugate or urine samples from biodistribution studies with suitable radioactive counts for the gamma counter was added to an Eppendorf microcentrifuge tube containing 0.5 mL of 1% BSA in 0.01 M PBS, pH 7.4. After adding 5 μL of 1 M NaI and 0.5 mL of 20% TCA, the solution was mixed on a Vortex Mixer and allowed to stand at 4^{0}C for 5 minutes. The mixture was then centrifuged in an Eppendorf Centrifuge for 4 minutes. The precipitate was washed twice with 0.01 M PBS. The pellet as well as the pooled supernatant were counted for radioactivity.

3.1.9 Radioiodination

3.1.9.1 Radioiodination Procedure

Antibody conjugates and control native antibody were radiolabeled with ^{125}I or ^{131}I by a mild oxidizing agent, Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril, Pierce Chemical Company, Rockford, USA) using the method originally described by Fraker and Speck (1978).

New glass tubes (5 mL) were cleaned by immersing in a 10% aqueous biodegradable detergent FL-70 (Fisher Scientific Company, Edmonton, Canada) at boiling temperature for 5 minutes. After cooling the tubes were rinsed with DD-H_2O and dried. Twenty five μL of dichloromethane containing 10 μg of fresh Iodogen was added to each tube and air dried at room temperature.

Iodination grade solutions of ^{125}I-NaI and ^{131}I-NaI, 4000 MBq/mL in 0.1 M NaOH were purchased from Edmonton Radiopharmaceutical Centre (ERC), Edmonton, Canada and were buffered with 0.05 M PBS, pH 7.4. Fifty to 100 μg (1μg/μL) antibody conjugates in 0.01 M PBS, pH 7.4, were added to the glass tube coated with Iodogen followed by 5 μL of the radioiodide solution (1.85 MBq to 3.7 MBq, 50 μCi to 100 μCi). The reaction was allowed to proceed at room temperature (25°C-28°C) for 20-30 minutes with occasional gentle agitation. The reaction was then stopped by transferring the reaction mixture to another glass tube containing 5 μL of 0.02 M aqueous NaI solution. The mixture was incubated for another 10 minutes and then subjected to separation by Sephadex gel chromatography.

3.1.9.2 Separation of Unreacted Radioiodide

Sephadex G-50 gel (fine) (Pharmacia, Sweden) was initially used for the separation. The gel was first allowed to hydrate in 0.01 M PBS overnight with approximately 4 mL bed volume per gram of the dry gel. Sephadex G-50 gel was used in later experiments. A column (0.8 x 14 cm) was packed with the swollen gel. Non-specific protein binding sites on the gel were saturated with 1 mg of a 1% BSA solution before application of the radioiodinated protein mixture. The reaction mixture was allowed to permeate into the gel bed and a small amount of buffer solution was used to complete the loading. The column was eluted with 0.01 M PBS buffer, pH 7.4. Aliquots of 0.5 mL were collected and 10 μL of each aliquot was counted for radioactivity in a Beckman 8000 Gamma Counter.

3.1.9.3 Quality Control of Radioiodinated Proteins

Protein fractions collected from a gel filtration column were analyzed by ITLC in methanol (MeOH)/H_2O 85/15 (v/v) solvent system for radiochemical purity. The ITLC strips were developed to 9 cm. The strips were cut into 1 cm sections and counted in a NaI(Tl) well-counter for gamma radioactivity. The protein bound radioactivity remained at the origin while the free radioiodide moved to the solvent

front. Percentage of protein bound radioactivity was calculated by the ratio of counts at origin to total counts (counts at solvent front plus counts at origin).

Radioiodination yield was determinated by ITLC before purification. Some batches of radioiodinated antibodies were subjected to TCA precipitation assay as well.

3.1.10 Animal Biodistribution Study in Normal Mice

ICR normal male mice, 25-35 g, were obtained from the University of Alberta Health Sciences Laboratory Animal Services. One to three μg of antibody conjugates in 0.1 mL of PBS, pH 7.4 or citrate buffer, pH 6.0 were administered into mice by tail vein injection. Syringes were counted before and after injection to yield the net injected dose. At various time intervals the mice were exsanguinated by cardiac puncture under CO_2 anesthesia. Tissues of interest were dissected and blotted to remove adhering blood. The blood and tissues were weighed in tared counting vials and counted for radioactivity in a Beckman 8000 Gamma Counter. Percentage of injected dose per gram wet tissues (% injected dose/g), per organ (% injected dose/organ) and standard deviation were calculated by a personal computer using Lotus 123 program.

For paired studies of biodistribution of the sialic acid antibody conjugate, ^{131}I labeled sialic acid conjugated hIgG and ^{125}I labeled native hIgG were coinjected into mice. A dual label gamma counting program was used with a Beckman 8000 instrument to account for crossover.

A decay correction for ^{111}In and ^{99m}Tc-labeled compounds was applied in the calculation of biodistribution studies and for all experimental measurements.

3.2 Preparation and Biological Behaviour of GalNAc and Galß(1,3)GalNAc (GalGalNAc) Antibody Conjugates

3.2.1 Preparation of Amino Derivative of GalNAc

Fig. 3.1 gives an outline of the synthetic strategy for the preparation of amino

derivative of GalNAc. The solvents used in this study were not further purified unless specifically indicated. GalNAc was purchased from Aldrich Chemical Company, USA. The phthalimide derivative **2** (in Fig. 3.1) was provided by Dr. K. R. Koganty, Biomira Inc. TLC was carried out on silica gel F-254 glass plates (Merck, Germany). Compounds were visualized by UV light and by spraying with 5% ninhydrin in ethanol (EtOH) solution for primary amino group detection. The existence of carbohydrate was monitored by spraying with 5% H_2SO_4 in EtOH solution followed by heating the plates. Solvent systems used in TLC and column chromatography were: (1) MeOH/$CHCl_3$ 1/9 (v/v); (2) $CHCl_3$/MeOH/H_2O 13/7/1 (v/v); (3) CH_2Cl_2; and (4) EtOAc

Four hundred and fifty mg (2.195 mmol) of GalNAc **1** (Fig. 3.1) and 1 g (4.25 mmol) of derivative of phthalimide **2** (Fig. 3.1) were suspended in 5 mL of DMF saturated with HCl. Reaction was carried out at 60°C for 4 hours. After completion the reaction mixture was washed with anhydrous ethyl ether (4 mL X 3). Solvent was evaporated and the residue was loaded onto a silica gel 60H (fine, Merck, Germany) column (4.5 x 25 cm), and eluted with solvent system (1) at a flow rate of 0.5 mL/min.

To 69 mg (0.16 mmol) of compound **3** (Fig. 3.1), 3 mL of EtOH/H_2O 1/1 (v/v) and 0.5 mL of hydrazine hydrate were added. The reaction mixture was stirred for 4 hours at 80°C. The crude product was purified by an aluminum oxide (neutral, Merck) column (1.8 x 23 cm) eluted with solvent system (2) and then with DD-H_2O. The purified compound was subjected to ^{1}H-NMR for characterization.

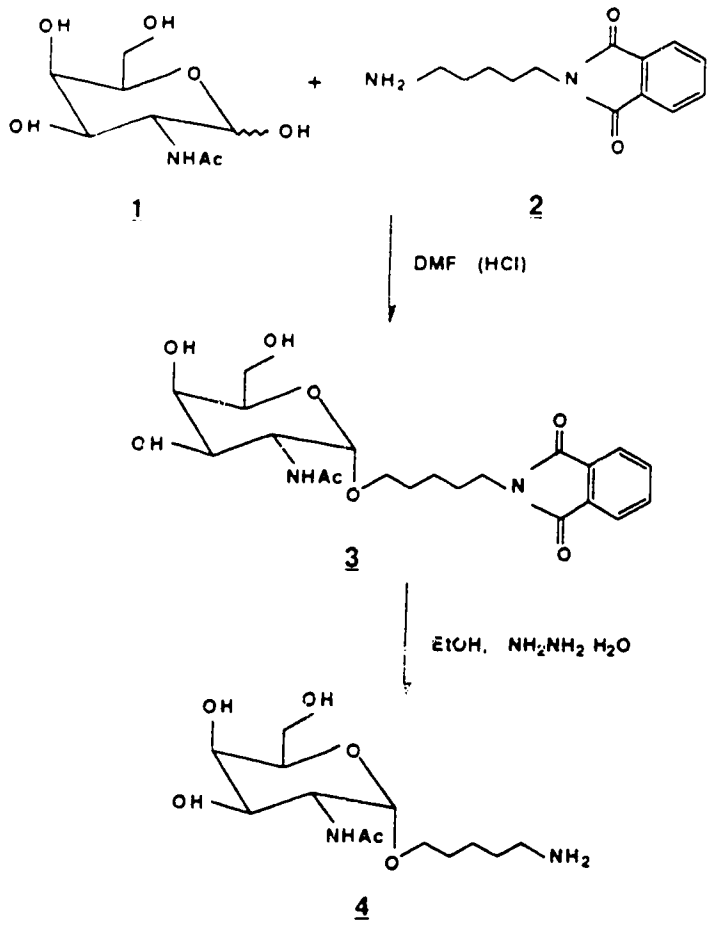


Fig. 3.1 Preparation of amino derivative of GalNAc

3.2.2 Distillation of the Fluoro-Linking Reagent

Hexafluoroglutaric acid dimethyl ester **5** (HFGDE, Fig. 3.2, provided by Biomira Inc.) with two active functional ester groups was used as a bifunctional agent. One ester group was reacted with the amino derivative of carbohydrates or other amino compounds. The other one was reserved for antibody conjugation. HFGDE was purified by distillation at 0.4 mm Hg and 41.5°C-44.5°C fractions were collected.

^{1}H-NMR ($CDCl_3$) and ^{19}F-NMR were performed for characterization.

3.2.3 Linking Reaction (Monosubstitution) of Fluoro Linking Reagent with Amino Derivatives of GalNAc and GalGalNAc

Amino derivative of GalGalNAc **6b** (Fig. 3.2) was provided by Dr. Koganty (Biomira Inc.). The linking reaction outline is illustrated in Fig. 3.2. For a typical reaction, 20 mg of amino derivatives of GalNAc and GalGalNAc were suspended in 3 mL of anhydrous MeOH and 0.5 mL of HFGDE. The reaction mixtures were stirred at 4°C for 2-3 hours and then the solvent was evaporated. The residue was washed thoroughly with anhydrous ethyl ether to remove excess linking reagent which, if present, would cause cross linking between IgG molecules. The ether layer was pipetted out and all remaining solvent was finally removed under vacuum. The resulting residue was utilized in the subsequent conjugation reaction. Linking compounds **7a** and **7b** (Fig. 3.2) were maintained in an anhydrous state and dissolved in DD-H_2O just before conjugation to proteins.

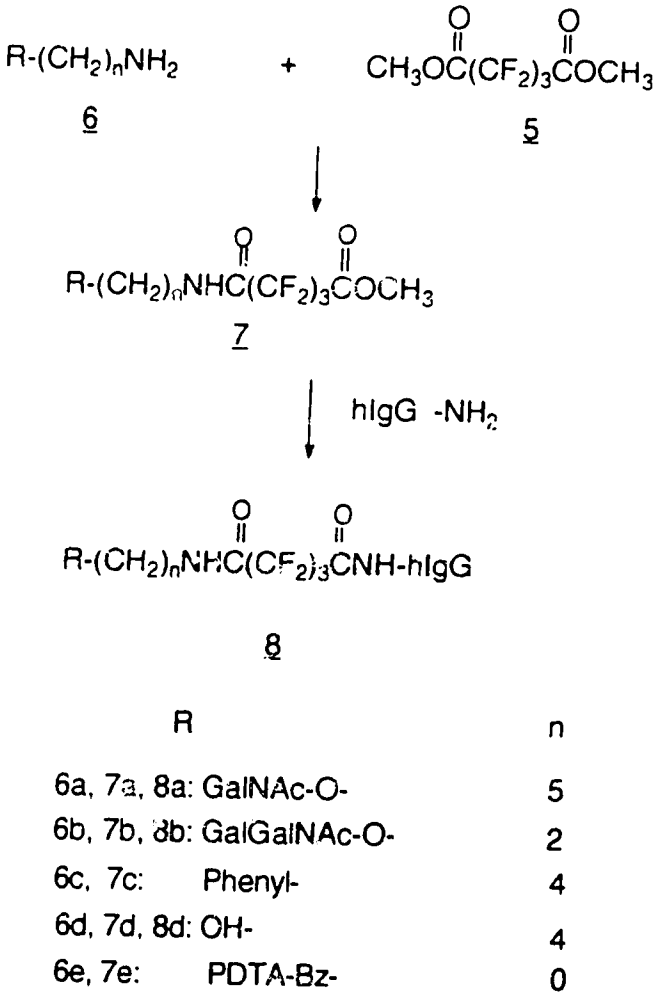


Fig. 3.2 Preparation of sugar-hIgG conjugates.

3.2.4 Linking Reaction of Fluoro Linking Agent with Two Non-Sugar Amino Compounds and a chelating agent, PAB-PDTA

Similar linking reactions were performed using two non-sugar amino compounds, 4-phenylbutylamine **6c** and 4-amino-1-butanol **6d** (Fig. 3.2) as controls in biodistribution studies to investigate the influence of the linking arm itself. To 400 μL of anhydrous MeOH, 0.04 mmole of the amines and 0.4 mmole of HFGDE were added. The reaction mixture was stirred at 4°C for 2-3 hours. Linking products **7c** and **7d** (Fig. 3.2) were purified by silica gel columns (70-230 Mesh, 1 x 20 cm) eluted with CH_2Cl_2 and ethyl acetate (EtOAc) respectively. TLC analyses were carried out with the same solvent systems as those for column chromatography. Compound **7c** (Fig. 3.2) has an R_f value of 0.63 in TLC developed by CH_2Cl_2. Compound **7d** has an R_f of 0.57 developed by EtOAc. ^{1}H-, ^{19}F-NMR were performed for the characterization.

A PAB-PDTA linking product with HFGDE **7e** (Fig. 3.2) was also obtained by a similar procedure. Twenty mg of PAB-PDTA was suspended in 2 mL of anhydrous MeOH and 240 μL of HFGDE (HFGDE/PDTA 22/1). The reaction mixture was stirred for 6 hours at 4°C. The solution became clear gradually. The solvent was removed under vacuum by a rotovap. Product **7e** (Fig. 3.2) was precipitated by addition of anhydrous ethyl ether. The precipitate was washed by anhydrous ethyl ether (6 x 3 mL) to remove excess HFGDE, and the solvent was finally evaporated under vacuum. Purified **7e** was dissolved in D_2O and subjected to 300 MHz ^{1}H-NMR and ^{19}F-NMR.

3.2.5 Study of the Hydrolysis of the Methyl Ester Bond in PDTA-linked Fluorinated Linking Reagent

Compound **7e** (Fig. 3.2) was dissolved in D_2O just before ^{1}H-NMR was taken. ^{1}H-NMR spectra were recorded at 3 minutes, 20 minutes, 1 hour, 2 hours, 4 hours and 6 hours. Hydrolysis of the methyl ester bond was monitored from the NMR spectra. By choosing an integer of aromatic protons (4H) per molecule as a

standard, the change of the integer of the methyl group protons (3H) relative to that of the aromatic protons was quantified as percentage of hydrolysis. A plot of percentage of hydrolysis at 20°C *vs* time was drawn. Half life of the hydrolysis was obtained from the plot as well.

3.2.6 Conjugation of GalNAc and GalGalNAc to hIgG

Conjugation of GalNAc and GalGalNAc to the amino group on the side chain of lysine or arginine of hIgG was carried out by reacting 5 mg of hIgG in 1 mL 0.05 M NaOAc buffer, pH 5.5 with various amounts of carbohydrate derivatives (**7a** and **7b**, Fig. 3.2) in 50-250 μL of DD-H_2O at 4°C overnight. Separation of conjugated hIgG from free sugar derivatives was carried out by passing the reaction mixture through a Sephadex G-50 gel filtration column (1 x 33 cm) eluted with DD-H_2O. The protein portion was collected and lyophilized. Protein concentrations were determined spectrophotometrically.

3.2.7 Conjugation of Fluoro Linking Product 7d to Antibody

Compound **7d** (Fig. 3.2) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10-20 μg/μL. The final concentration of DMSO was less than 5% in the solution to avoid precipitation of proteins. The concentration of hIgG was 3.3 mg per mL in NaOAc buffer, pH 5.5. Starting molar ratio of linking product **7d** to hIgG was 50:1 or 80:1. The reaction was carried out at 4°C for 12 hours.

Conjugated hIgG was purified by a Sephadex G-50 gel column (1 x 36 cm) as described in section 3.1.3.

3.2.8 Estimation of the Number of Sugars per Antibody Molecule by ^{19}F-NMR Signals

NMR samples were prepared by adding 10 μL (21 μg) of NaF as an internal standard to sugar antibody conjugate solutions. Two ^{19}F signals from six fluorine atoms of one HFGDE molecule and one ^{19}F signal from standard NaF were

integrated and compared. The following equations were used in the calculation.

$F_4 = (F_1/6F_2)(F_3)$ (3.5)

$F_5 = F_4/P$ (3.6)

Where:

F_1 = total integer from two signals of fluorine linking arm

F_2 = integer from NaF

F_3 = number of moles of fluorine atom in NaF added as an internal standard

F_4 = total number of moles of fluorine linking arm (sugar) in sample

P = number of moles of antibody sugar conjugate in sample

F_5 = number of sugars per antibody conjugate molecule

3.2.9 Biodistribution Study of Sugar Conjugates in Normal ICR Mice

The antibody conjugates were examined for aggregate formation using a Sepharose CL-4B column as described in section 3.1.4. Preparations with less than 10% aggregates for sugar conjugates and less than 6% for amino compound conjugates were chosen for the biodistribution study.

The conjugates were radioiodinated by the method described in section 3.1.9. Radioiodinated 4-amino-1-butanol-hIgG conjugate and native hIgG served as two controls. The differential tissue distribution was performed using normal male ICR mice as described in section 3.1.10. Purified radioiodinated conjugates with greater than 95% radiochemical purity, as determined by ITLC, were administered to each mouse with about 18.5 KBq (0.5 μCi) of radioactivity, 1-2 μg of labeled conjugates. A group of 3 to 4 mice were dissected at time points of 2 min, 15 min, 1 hour, 6 hours, 12 hours and 24 hours.

3.3 Preparation of Sialic Acid-hIgG Conjugate

3.3.1 Preparation of Aldehyde Derivative of Sialic Acid

A linking derivative of sialic acid containing an aliphatic aldehyde functionality was utilized in the conjugation of sialic acid to hIgG (Fig. 3.3).

Allyl derivative of sialic acid **9** (Fig. 3.3) was provided by Dr. N. Selvaraj (Biomira Inc.). Aldehyde derivative of sialic acid **10** (Fig. 3.3) was obtained by ozonolysis of compound **9** (Fig. 3.3) followed by reduction using dimethyl sulfide (Me_2S). For ozonolysis, 50 mg of compound **9** (Fig. 3.3) was dissolved in 10 mL of MeOH. Ozone gas (O_3) was bubbled through the reaction solution for 15-20 minutes in an ice bath. The progress of the reaction was monitored by TLC developed in $CHCl_3$/MeOH/H_2O 13/7/1 (v/v) and visualized by spraying with 5% resorcinol in EtOH solution. The starting material **9** (Fig. 3.3) had an R_f value of 0.26 while the product **10** (Fig. 3.3) remained close to the origin with an R_f 0-0.09 in this solvent system. Excess O_3 was bubbled out with dry nitrogen (N_2) and followed by addition of 0.5 mL of Me_2S. The reaction mixture was stirred for an additional hour. Excess Me_2S was removed by bubbling N_2 through the solution. The crude product after removing the solvent was loaded onto a Bio-Gel P-2 (1 x 35 cm) column eluted with DD-H_2O at a flow rate of 1 mL/10 min. One mL of aliquot was collected. The appropriate fractions were then pooled and freeze-dried. Purified compound **10** (Fig. 3.3) was obtained in 67% yield.

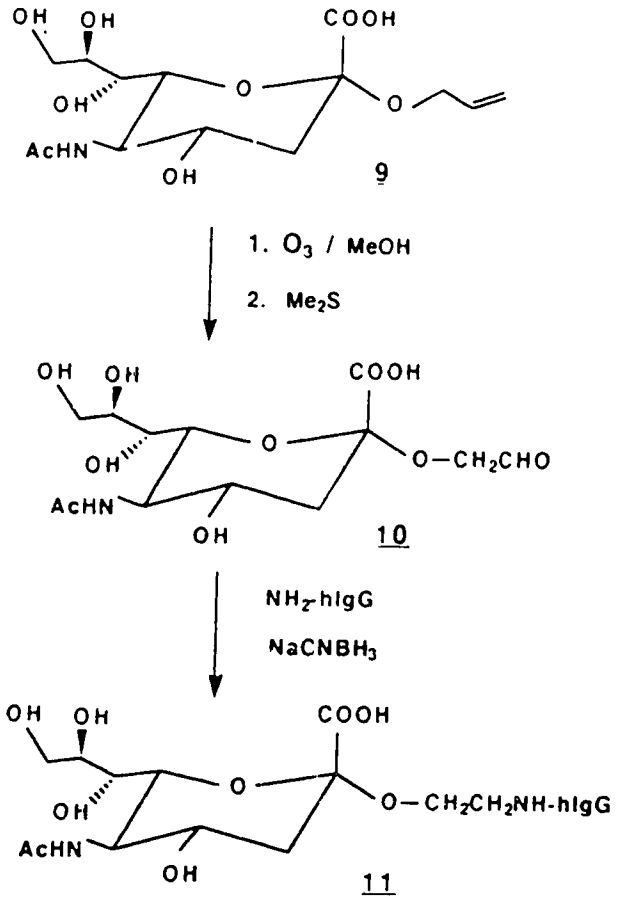


Fig. 3.3 Preparation of sialic acid-hIgG conjugate

3.3.2 Preparation of Sialic Acid hIgG Conjugate

The aldehyde derivative of sialic acid was subsequently coupled to hIgG at a molar ratio of 100:1 under reducing conditions by addition of $NaBH_3CN$ (10 mM) in a phosphate buffer, pH 7.5 for various time periods. The same reaction procedure was also performed for the control by using sialic acid instead of its aldehyde derivative.

Compound **11** was purified by a Sephadex G-50 column. Aggregate formation was monitored by a Sepharose CL-4B column as described in section 3.1.4.

3.3 3 Estimation of Sialic Acid Residues per hIgG Molecule

3.3.3.1 Preparation of Resorcinol Reagent

A stock resorcinol solution was prepared by dissolving 2 g of resorcinol in 100 mL of DD-H_2O. This solution was stable for a few months when stored in a refrigerator. A working solution was made by adding 10 mL of the stock solution to 80 mL of concentrated HCl and 0.25 mL of 0.1 M copper sulphate. The volume was then made up to 100 mL with DD-H_2O. This solution was prepared at least 4 hours prior to use and was stable for a week at 4°C (Svennerholm, 1957).

3.3.3.2 Analysis Procedures

Sialic acid (Sigma Chemical Co., USA) was carefully weighed in a 0.001 mg sensitive balance (Mettler, AE163, Fisher Scientific Co., Edmonton, Canada) and dissolved in DD-H_2O to a concentration of $\mu g/\mu L$. A standard curve was constructed by pipetting 10, 20, 30, 40, 50 μg of sialic acid slution ($\mu g/\mu L$) into KIMAX glass test tubes (1.5 x 12 cm). The volume was then made up to 1 mL with DD-H_2O. One mL of sialic acid conjugated antibody containing approximately 10 μg to 50 μg of sialic acid and 5 mg of native antibody in 1 mL were assayed along with the standards. This was followed by addition of 2 mL of the resorcinol working solution. The test tubes were capped and heated in a boiling water bath for 15 minutes. After cooling the reaction mixture in an ice bath, 2 mL of n-butanol/tertbutyl acetate

15/85 (v/v) was added to each tube and mixed with a Vortex mixer to extract the colour produced into the organic phase. The absorbance at 580 nm of the organic layer was determined after 10-15 minutes.

Estimation of sialic acid residues per hIgG molecule was based on the fact that an average of about 1 sialic acid residue was originally present per native hIgG molecule (Parekh, et al., 1985).

3.3.4 Biodistribution of Sialic Acid Conjugate in Normal Male ICR Mice

The sialic acid-hIgG conjugate with less than 5% aggregates was radioiabeled with ^{125}I. In the case of a paired test, approximately 1 μg, 5 μCi of ^{131}I labeled sialic acid conjugate was coinjected with ^{125}I-labeled native hIgG into a mouse by tail vein injection. The cross over of the radioactivity from ^{131}I to ^{125}I was calibrated with a dual label counting program. Other conditions were the same as those mentioned in section 3.1.10.

3.4 Periodate Oxidation of Antibodies

3.4.1 Oxidation and Purification Procedures

Oxidation of the oligosaccharide moiety of antibodies was carried out by adding freshly prepared aqueous sodium periodate solution to an antibody solution in sodium acetate buffer, pH 5.2 for hIgG, and pH 6.0 for MAb 155H.7. The protein concentrations ranged from 3.3 mg to 5 mg per mL. The final concentration of sodium periodate was 10 mM or 30 mM. Oxidation reactions were performed at room temperature (25°C-30°C) or 4°C for 30 minutes usually in the dark. Some exceptions will be indicated in the related text. The mixture was then passed through a Sephadex G-50 column (1 x 38 cm) or a Bio-Rad P-6DG column (1 x 40 cm) and eluted with 0.01 M citrate buffer, pH 6.0 or 0.01 M NaOAc buffer, pH 5.2. The eluent was monitored by UV absorbance at 280 nm by an LKB Bromma UVICORD SII Monitor. The oxidized protein fraction was collected and used directly for the conjugation to hydrazine compounds. For the conjugation of the

oxidized antibody to the chelating agent PAB-PDTA, the collected protein portion was lyophilized (2 to 3 hours) and then reconstituted with DD-H_2O to a required concentration of 2 to 3 mg/mL and immediately used for the conjugation reaction.

3.4.2 Study of Storage of Oxidized Antibody

The oxidized antibody was analyzed by a Sepharose CL-4B column and HPLC with a TSK 3000SW size-exclusion column for aggregate and oligomer formations. The tests were performed with the fresh preparation and the preparations stored at 4°C for a few days up to 15 days. The effect of prolonged storage, or freeze-drying periods of the oxidized antibodies from 8 hours to 15 hours, on subsequent conjugation reactions was also examined.

3.5 Linkage of 2,4-Dinitrophenylhydrazine (DNPH) to the Carbohydrate Moiety of Antibodies and GalGalNAc Conjugated HSA (HSA-Tα)

DNPH was used to react with the aldehyde groups resulting from periodate oxidation of antibodies.

3.5.1 Estimation of the Number of DNPH per Protein Molecule

A UV spectrum of DNPH in 0.01 M PBS buffer, pH 7.2 at a concentration of 18 μg/mL was measured. Molar extinction coefficient of DNPH and percentage of the cross absorbance at 280 nm from that at 360 nm was calculated. This cross over was deducted from the absorbance at 280 nm in the calculation of the protein concetration of DNPH-protein conjugates.

DNPH-protein conjugate solution collected from a gel filtration column was determined for absorbance at 280 nm and 360 nm. Calculation of the protein concentration was based upon the UV absorbance at 280 nm with $\epsilon^{1\%} = 14$ for antibody, $\epsilon^{1\%} = 6.6$ for HSA and subtraction of the contribution from DNPH at 280 nm, which was about 40% of its absorbance at 360 nm. To calculate the concentration of DNPH residues on DNPH-protein conjugates a molar extinction coefficient at 360 nm, $\epsilon = 1.37 \times 10^4$ was used. A blank was obtained by mixing

DNPH with native antibody under the same reaction conditions. The following formulae were used in the calculation:

$$\text{Number of DNPH/Ab} = \frac{\text{Molar concentration of DNPH}}{\text{Molar concentration of Ab}}$$

$$= \frac{A_{360nm}/\epsilon_{360nm,DNPH}}{(A_{280nm}-(A_{360nm}x0.4))/\epsilon_{280nm,Ab}} \quad (3.7)$$

Where:

$\epsilon_{360nm,DNPH} = 1.37 \times 10^4$

$\epsilon_{280nm,Ab} = 14 \times 150{,}000/10 = 2.1 \times 10^5$

($\epsilon^{1\%}_{Ab} = 14$, $MW_{Ab} = 150{,}000$)

Similarly, for the calculation of the number of DNPH/HSA-Tα, a $\epsilon_{280nm,HSA\text{-}T\alpha}$ value of 4.62×10^4 was used to replace $\epsilon_{280nm,Ab}$ in equation (3.7).

3.5.2 Effect of Starting Ratio of DNPH to hIgG

Twenty to thirty μL of the oxidized antibody collected from a gel filtration column was subjected to protein concentration determination. The remaining portion of the solution was then aliquoted. To 250-300 μg of the oxidized hIgG, a certain amount of NaOAc or citrate buffer (0.01 M, pH 6.0) was added to make up the volume to 700 μL. A calculated amount of DNPH in DMSO (0.05 M) was added carefully to the protein solution with a total final concentration of DMSO to be less than 5%. Ratios of DNPH to hIgG up to 300 were tested in this experiment but protein precipitation was observed at a ratio higher than 250. The reaction mixture was incubated at 4°C for 15 hours. Excess DNPH was removed by passage of the reaction mixture through a Sephadex G-50 column (0.8 x 18 cm) eluted with 0.01 M PBS buffer, pH 7.2. The protein portion was collected and subjected to measurement of absorbance simultaneously at 280 nm and 360 nm. Batches of samples were purified by setting up a number of columns which had been calibrated

for void volume, and the protein fractions were collected at the void volume without the use of an UV monitor.

3.5.3 Effect of Linking Reaction Time

The oxidized hIgG was aliquoted and a starting ratio of 180 of DNPH to hIgG was used. Reactions were carried out in duplicate at different time intervals. Other procedures were the same as those described in section 3.5.2.

3.5.4 Effect of Concentration of Sodium Periodate

Sodium periodate concentrations, 0.0, 0.1, 1.0, 10.0, 30.0 mM were tested while other parameters, such as starting ratio of DNPH/hIgG, linking reaction time, reaction temperature were held constant.

3.5.5 Periodate Oxidation of HSA, as a Model Non-glycoprotein

HSA, as a non-glycoprotein, was used to investigate the possible periodate oxidation of some amino acids on proteins under the same reaction conditions.

3.5.6 Linearity

3.5.6.1 Theoretical Assumptions

A neoglycoprotein, HSA-Tα, with a known amount of disaccharide, GalGalNAc (Tα) analyzed by conventional phenol sulphuric acid method was provided by Biomira Inc. for this study.

An equation can be drawn by combining the constants in equation (3.7):

$$\#\text{ of DNPH/hIgG} = 15.33 \times \{A_{360nm}/(A_{280nm}-(A_{360nm}\times 0.4))\}$$

$$= 15.33 \times \text{S value} \qquad (3.8)$$

and $$\#\text{ of DNPH/HSA-T}\alpha = 3.73 \times \{A_{nm}/(A_{280nm}-(A_{360nm}\times 0.4))\}$$

$$= 3.73 \times \text{S value} \qquad (3.9)$$

Where: $\text{S value} = A_{360nm}/(A_{280nm}-(A_{360nm}\times 0.4))$

A straight line would be obtained by plotting the number of DNPH/protein

vs S value with a slope of around 3.73 using HSA-Tα with a different but known amount of sugars per molecule, if the method were reproducible.

3.5.6.2 Experimental Procedures

According to the results in the study of conjugation procedure a set of parameters were selected to investigate the reproducibility of the method. Periodate oxidation of a HSA-Tα solution was performed in 30 mM of $NaIO_4$, at 4°C for 1 hour in the dark. The starting ratio of DNPH to protein in conjugation reaction was 180 and the reaction mixture was incubated at 4°C for 15 hours.

A series of dilutions of HSA-Tα (3 mg/mL) with HSA (3 mg/mL) in NaOAc buffer solution, at the ratios of HSA-Tα to HSA of 0.3, 1, 3, were made. The resulting HSA-Tα solution mixture, along with the original HSA-Tα solution were oxidized with periodate and conjugated with DNPH in duplicate under conditions mentioned above. Data were calculated and graphed using a Cricket graph program in a MacIntosh computer.

3.5.6.3 An Indirect Method to Analyze the Number of Sugar Residues on Glycoprotein Molecules

The number of saccharides on a glycoprotein that could possibly be oxidized by periodate can be estimated theoretically according to the corresponding carbohydrate structures. The number of DNPH residues per glycoprotein molecule is then correlated to the theoretical number of saccharides on the glycoprotein. Consistent correlations in this matter were observed using glycoproteins, hIgG, MAb 155H.7 and HSA-Tα.

3.6 Effect of Structural Modifications of Carbohydrate Moiety of hIgG and MAb 155H.7 on Biodistribution in Mice

3.6.1 Chemical Modifications

Periodate oxidation of antibodies was conducted in 10 mM of $NaIO_4$, 25°C

for 30 minutes in the dark. The oxidized antibody collected from a gel column was aliquoted and the protein concentration was determined. One aliquot was reduced by adding $NaCNBH_3$ to a final concentration of 10 mM. One portion was conjugated with 100 fold excess tyrosine hydrazide. DNPH conjugation was carried out by addition of 0.05 M DNPH in DMSO solution to one aliquot of oxidized antibody at a ratio of 150:1. The reaction mixtures were then incubated at 4°C for 15 hours and purified by a Sephadex G-50 (1 x 35 cm) column. Purified conjugates were checked for aggregate formation using Sepharose CL-4B chromatography. The protein concentration of each was determined as well.

3.6.2 Early Tissue Biodistribution in Normal Mice

The modified antibodies were radiolabeled with radioiodide-^{125}I by the Iodogen method. Biodistribution studies were performed as those described in section 3.1.10. Blood clearance and tissue uptake data of radioiodinated antibody conjugates and radioiodinated native antibody were compared in terms of percent injected dose per gram of wet tissue (inj. dose/g) at 15 minutes post injection (PI).

3.7 Purification and Chelation Study of the Novel Chelating Agent para-nitrobenzylpropylenediaminetetraacetic Acid (PNB-PDTA)

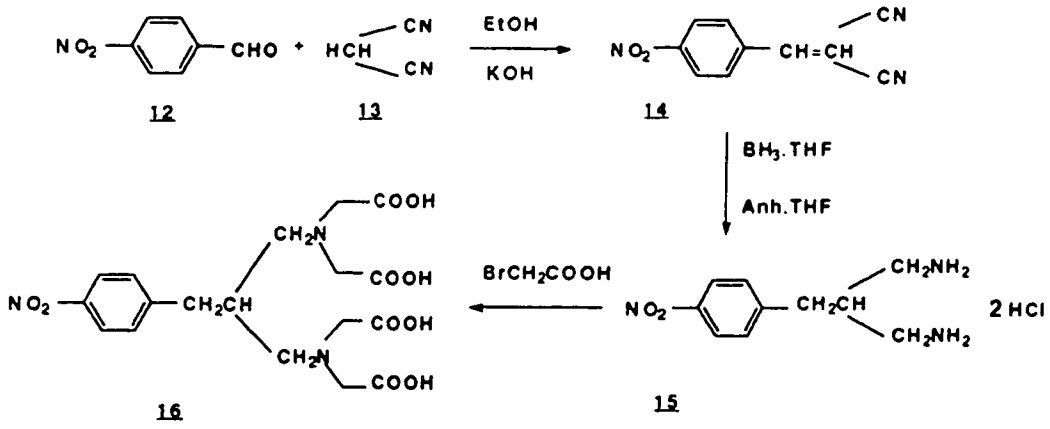


Fig. 3.4 Synthetic outline of PNB-PDTA (Dr. R. Gaudreault)

3.7.1 Purification of PNB-PDTA

Crude product of PNB-PDTA synthesized according to the scheme in Fig. 3.4 was suspended in DD-H_2O and dissolved by dropwise addition of 7 M sodium hydroxide solution. A brown reaction mixture was loaded onto an AG 1-X4 anion exchange (formic acid type, Bio-Rad Laboratories (Canada) Ltd.) column (4 x 40 cm) eluted with gradient formic acid solution from 0-7 M (total volume of 2 liters). Fractions were collected by a fraction collector. The product was further purified by a Bio-Gel P-2 column (Bio-Rad, 1 x 40 cm) eluted with DD-H_2O.

3.7.2 Chelation with ^{111}In

Chelation studies were carried out on the purified fractions. ^{111}Indium chelation was achieved by adding ^{111}In in 0.05 M HCl solution to an aqueous chelate solution in 0.1 N HCl. A small aliquot of the reaction mixture was spotted on TLC silica gel plate after a 20 minute reaction. The plate was developed with 10% NH_4OAc/MeOH 1/1 (v/v). The ^{111}In-chelate moved to the front of the TLC plate and $^{111}InCl_3$ stayed in the origin.

3.7.3 Chelation with ^{99m}Tc

Formamidine sulfinic acid (FSA) was used as a reducing agent in ^{99m}Tc chelation study. Ten mg of FSA was suspended in 1 mL DD-H_2O at room temperature for 20 minutes and filtered by passage through a 0.22 μm filter. The resultant solution containing dissolved FSA was used for reduction of pertechnetate solutions. No attempt was made to determine the amount of dissolved FSA. Analysis was performed by paper chromatography (Whatman No.1) in NaOAc (0.1 M, pH 6)/CH_3CN 10/1 (v/v) development system. The effect of the amount of reducing agent on the labeling yield was carried out by adding relative volumn of FSA solutions. The effect of solution pH and reaction temperature on the chelating efficiency were also investigated.

3.8 Preparation and Chelation Study of Novel Chelating Agent p-NH_2-Bz-PDTA (PAB-PDTA)

3.8.1 Hydrogenation of PNB-PDTA

The chelating agent PNB-PDTA was also synthesized by Dr. Vincent Guay in this laboratory. Characterization of this new compound will be documented elsewhere. The amino derivative of PNB-PDTA, PAB-PDTA was obtained by hydrogenation of PNB-PDTA using Pd/C (Aldrich Chemical Company, Inc., USA) as a catalyst. One hundred mg of PNB-PDTA and 21 mg of 10% Pd/C were suspended in 5 mL of DD-H_2O. The suspension was precooled in an ice bath and 0.83 mL of 1.2 M NaOH (metal free, Merck, Germany) was added slowly. An atmosphere hydrogenation apparatus system and reaction flask were evacuated under vacuum and flushed with H_2. The hydrogen pressure was set up to 1 atmosphere and the changes of the hydrogen pressure during reaction were monitored. The reaction was stopped after 5 hours when hydrogen pressure was stabilized. The reaction mixture was filtered through layers of cotton and sea sand in a pipette and the product was lyophilized.

Seven mg of PAB-PDTA dissolved in D_2O (Aldrich) was subjected to ^{1}H-NMR (300 MHz).

3.8.2 Chelation of PAB-PDTA with ^{111}In

An ^{111}In-citrate solution was prepared by 20 fold dilution of the $^{111}InCl_3$ with 0.1 M citrate buffer, pH 6.0.

The free chelate, PAB-PDTA, was labeled with ^{111}In by addition of ^{111}In-citrate to an aqueous solution of chelate at room temperature. The percentage of ^{111}In-chelate was checked by ITLC developed with a solvent system, 10% NH_4OAc/MeOH 1/1 (v/v). The ^{111}In-chelate moved to the solvent front while ^{111}In-citrate stayed at the origin.

3.9 Conjugation of PAB-PDTA to the Carbohydrate Moiety of Antibodies

3.9.1 Conjugation of the Chelating Agent, PAB-PDTA

Polyclonal hIgG and monoclonal antibody 155H.7 were used in this study. Periodate treatment of antibodies was the same as that described before. The oxidized antibodies were eluted from a gel filtration column (Sephadex G-50 or Bio-Rad P-6DG, 1x35 cm) with 0.01 M citrate buffer, pH 6.0. The protein portion was collected and lyophilized within 2 to 3 hours. The lyophilized antibodies were reconstituted with DD-H_2O to the concentrations of 2 to 3 mg/mL.

The chelate, PAB-PDTA, at predefined molar ratios, was added to the oxidized antibody solution and followed by the addition of $NaCNBH_3$ to a final concentration of 10 mM. The reaction was conducted at 4°C for varying time intervals. The reaction mixture was again passed through a gel filtration column (Sephadex G-50 or Bio-Rad P-6DG 1x35 cm) eluted with 0.01 M citrate buffer, pH 6.0 to separate free PDTA and $NaCNBH_3$ from PDTA-conjugates. The protein peak was collected and lyophilized.

The same procedures as those described above were applied except that the reduction by $NaBH_3CN$ was omitted for the non-reduced conjugation tests.

3.9.2 One Step Conjugation

3.9.2.1 Study of Periodate Oxidation of Chelate, PAB-PDTA

Periodate oxidation of PAB-PDTA was achieved by the addition of aqueous $NaIO_4$ solution to an aqueous solution of PAB-PDTA to a final concentration of 10 mM of $NaIO_4$. The reaction was allowed to proceed at 4°C in the dark for 30 min or longer depending on the nature of the investigation. The oxidized PAB-PDTA was separated from excess sodium periodate by passing the reaction mixture through a Bio-Rad P-6DG gel filtration column (1 x 43 cm) eluted with DD-H_2O at a flow rate of 0.5 mL/min. The eluent was monitored by UV absorbance at 280 nm using an LKB Bromma UVICORD SII Monitor. The oxidized PAB-PDTA (first

peak) was collected and freeze-dried. The lyophilized product with a light brown color was subjected to 300 MHz ^{1}H-NMR (in D_2O) and analysed by TLC using silica gel F-254 plate (Merck, Germany). The development systems used in TLC were (1) EtOH/NH_4OH 4/1 (v/v); (2) CH_3CN/H_2O 7/3 (v/v). The UV light absorbance and then 5% ninhydrin in ethanol spraying were used for the detection of the compounds.

3.9.2.2 One Step Conjugation of PAB-PDTA to Antibodies

One step conjugation was simply carried out by addition of an aqueous solution of $NaIO_4$ to the antibody solution (3.3 mg to 5 mg per mL in sodium acetate buffer, pH 5.2) followed by addition of the chelate, PAB-PDTA. The final concentration of $NaIO_4$ was 10 mM. Ratios of the chelate to antibody added were 10 to 50. The reaction was allowed to proceed in the dark at 4°C for 30 minutes.

Separation of the free chelate and $NaIO_4$ from the conjugate was achieved by a gel filtration (Sephadex G-50 or Bio-Rad P-6DG) column (1 x 40 cm) eluted with 0.01 M citrate buffer, pH 6.0.

3.9.3 Radiochelation of PDTA-antibody conjugate with ^{111}In

3.9.3.1 ^{111}In-labeling of Antibody Conjugates

All glass and plastic vials and containers were washed with H_2SO_4/HNO_3 1/1 (v/v) solution and rinsed thoroughly with DD-H_2O in order to remove trace amounts of metallic contamination. The buffers and the chelate solution were passed through a Chelex 100 gel (BioRad, Canada) column for the same purpose.

$^{111}InCl_3$ (Nordion International Inc., Canada) was diluted with 0.1 M citrate buffer, pH 6.0, to about 111-185 KBq (3-5 μCi) per μL. ^{111}In labeling of the PDTA-antibody conjugate was achieved by adding ^{111}In-citrate to the antibody conjugate in a citrate buffer, pH 6.0. Usually, ^{111}In-citrate was added to give a specific activity of 3.7-11.1 KBq (0.1-0.3 μCi) per μg of antibody conjugate. The higher specific activity could be obtained with a fresh preparation of $^{111}InCl_3$.

The percentage of protein bound ^{111}In was assayed by ITLC developed with acetone/saline 16/1 (v/v), pH 2.0, and cellulose TLC in MeOH, pH 2.0 (pH was adjusted with 0.1 M HCl). The ^{111}In-bound protein stayed at the origin while ^{111}In-citrate migrated up to a R_f value around 0.6-0.7. A Sephadex G-50 column (1 x 20 cm) and HPLC (TSK 3000SWXL column, eluted with 0.01 M PBS, pH 7.2) were utilized for the assay for selected samples as well.

3.9.3.2 Estimation of the Number of Chelates per Antibody Molecule

Estimation of the number of chelates on an antibody molecule was achieved by ^{111}In-labeling of a mixture containing a known amount of PNB-PDTA and a known amount of PDTA-antibody conjugate which had previously been separated from free chelate by a gel filtration column. The analysis of the percentage of the protein bound-^{111}In (P% in equation 3.10) was performed by Sephadex G-50 gel chromatography for selected samples.

The method used in the calculation was as follows:

$$\text{The number of PDTA residues/antibody conjugate} = P\% \times R / (1 - P\%) \qquad (3.10)$$

Where:

P% = percentage of the protein bound-^{111}In

R = ratio of PNB-PDTA added to antibody conjugate

Routine analysis to obtain P% was usually conducted by ITLC in a solvent system of acetone/saline 16/1 (v/v), pH 2.0 adjusted by 0.1 M HCl. The ITLC assay was chosen for routine use because of the simplicity. However, it was found that there was non-specific binding between ^{111}In and oxidized antibody analyzed by ITLC and cellulose TLC. Therefore, the percentage of protein-bound ^{111}In (P% in equation 3.10) was corrected by subtraction of the non-specific bound ^{111}In of the oxidized antibody itself from the percentage of protein bound radioactivity of PDTA-antibody conjugate labeled under the same conditions.

3.9.3.3 Transchelation study of ^{111}In-Labeled Conjugates

Stability of the ^{111}In-labeled conjugates was determined by a transchelation study whereby 50 μg of purified antibody conjugates were labeled with ^{111}In-citrate. After 15 minutes a 50 or a 100 fold excess free PAB-PDTA or EDTA was added to challenge the chelated Indium. Samples were incubated in citrate buffer, pH 6.0 at 25^{0}C and analyzed by ITLC.

The non-specific binding of ^{111}In onto oxidized antibody was examined by adding ^{111}In-citrate to an oxidized antibody solution and assayed by Sephadex G-50 chromatography and ITLC as well.

3.9.3.4 Biodistribution of ^{111}In-Labeled PDTA-Antibody Conjugate in Normal Mice

^{111}In-labeled PDTA-antibody conjugates with more than 98% of protein bound radioactivity as assayed by ITLC, was administered into ICR male mice (25-30 g) *via* a tail vein injection. About 18.5 KBq (0.5 μCi), 3 μg of labeled conjugate was given to each mouse. All other procedures were the same as those described in section 3.1.10.

4. RESULTS AND DISCUSSION

4.1 Determination of Protein Concentration

A standard curve of human IgG (in the range of 20 μg/mL to 150 μg/mL) was prepared by serial dilutions of the antibody solution and measurement of absorbance at 280 nm using an LKB PU 8700 Series UV/Vis Spectrophotometer. A linear regression correlation coefficient (r) of 0.99 of the standard curve demonstrated the reproducibility of the analytical method (Fig. 4.1a). From the linear regression equation, a percent extinction coefficient ($\epsilon^{1\%}$) of 13.99 was calculated. This agreed well with the known extinction coefficient of gamma immunoglobulins, $\epsilon^{1\%}$ is around 14 (Williams, et al., 1968). Use of a different spectrophotometer, HP 8452A Diode Array spectrophotometer, gave a similar standard curve with an observed $\epsilon^{1\%}$ value of 13.8 (Fig. 4.2b). Thus, the protein concentration, in all relevant experiments, was determined using either of the two spectrophotometers by UV absorbance at 280 nm.

Protein recovery from a Sephadex G-50 gel filtration column was evaluated by determining the concentration of the protein before and after column separation. An average recovery of 85.5% (S.D. 1.8, n=4) was obtained and was used in the calculation of the administered protein dose in animal studies.

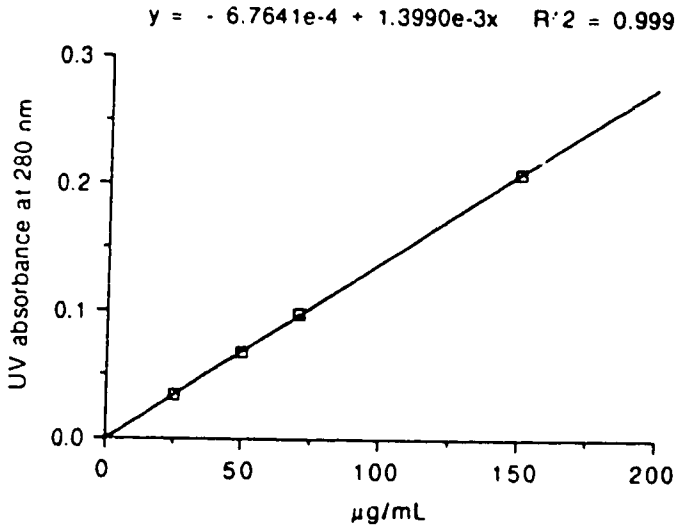


Fig. 4.1a A standard curve of hIgG concentration determined by UV absorbance at 280 nm using a LKB PU 8700 Spectrophtometer

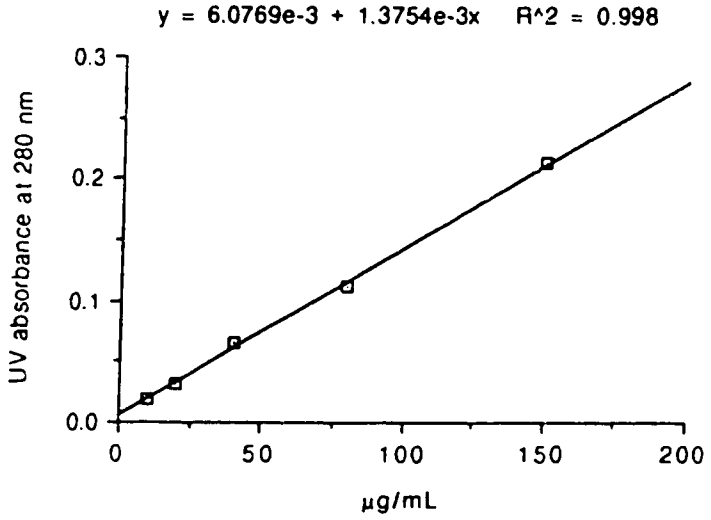
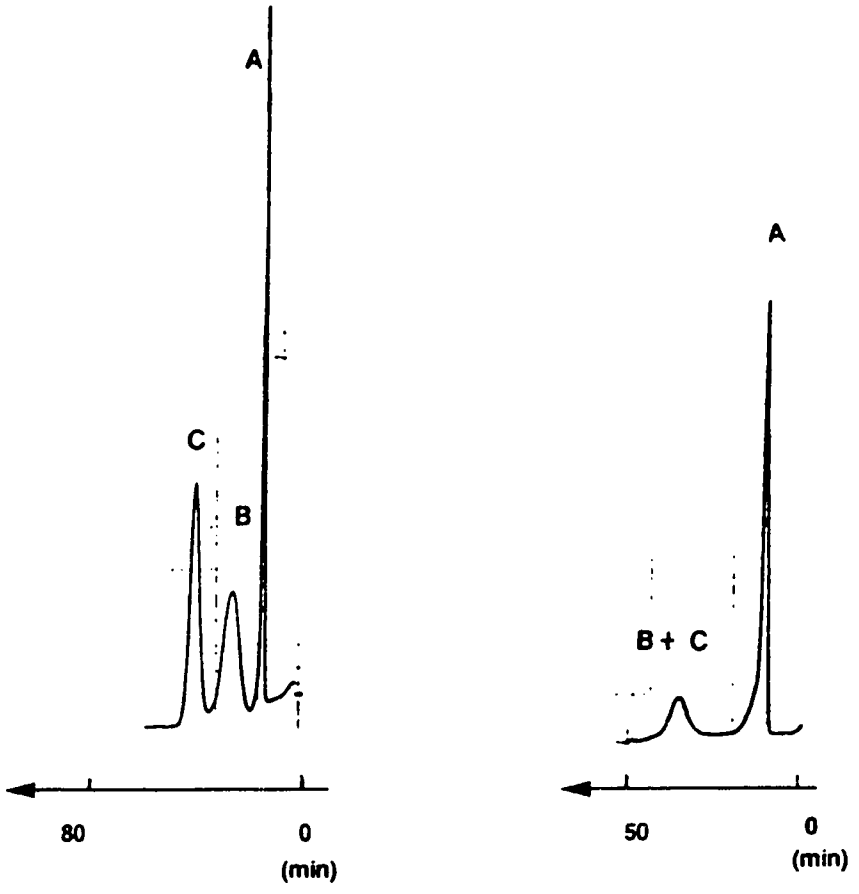


Fig. 4.1b A standard curve of hIgG concentration determined by UV absorbance at 280 nm using a HP 8452A Diode Array Spectrophotometer

4.2 Separation of Small Molecules from Conjugation Mixture by a Gel Filtration Column

Sephadex G-50 gel was used in the initial experiments. Later Bio-Rad P-6DG gel was also used in the separation. Both gels have good separation ability to remove small molecules from protein solutions. In one experiment a mixture consisting of hIgG, $NaIO_4$ and PAB-PDTA in 0.01 M citrate buffer was applied to the columns. Elution with the same buffer resulted in the separation of 2 peaks when Sephadex G-50 was used. The use of Bio-Rad P-6DG gel, on the other hand, gave a resolution of 3 peaks. The elution patterns are given in Figure 4.2.



Bio-Rad P-6DG Sephadex G-50

Fig. 4.2 Comparative separation of a conjugation mixture by Sephadex G-50 and Bio-Rad P-6DG chromatography (peak A: PDTA-hIgG conjugate, peak B: free PDTA, peak C: $NaIO_4$)

It can thus be concluded that Bio-Rad P-6DG gel is better suited to fractionate the small molecules but with prolonged elution time (60 minutes *vs* 50 minutes). In addition, Bio-Gel P-6DG gel is a synthetic porous poly-acrylamide material which will not support microbial growth because of the nature of the material. Synthetic gels are also more resistant to the action of reducing and oxidizing reagents, such as $NaCNBH_3$ and $NaIO_4$ which are to be separated from various mixtures used throughout this investigation.

4.3 Quality Control of the Formation of Aggregates during Manipulation of Antibodies

Apoferritin, gamma-globulin and BSA were used to calibrate the Sepharose CL-4B column (1 x 43 cm). The retention time of these standards as well as the aggregate of a polyclonal hIgG solution are shown in Table 4.1. A 20-minute difference in retention time between the IgG and its aggregate form was satisfactory for our analysis. On the other hand, the dimer of the antibody molecule with a molecular weight close to that of apoferritin did not exhibit good separation from its monomer form by the Sepharose CL-4B column. However, an observed difference in retention time of 7 minutes between the dimer and the monomer form allowed an indication of the presence of the dimer in the reaction mixture. The application of this technique for the separation and determination of hIgG aggregates, and those obtained after periodate oxidation of hIgG and PDTA-conjugated hIgG is given in Table 4.2. Fig. 4.3 depicts the separation of aggregated native hIgG from a solution containing 150 μg/100 μL 0.01 M PBS buffer.

Table 4.1 Calibration of the Sepharose CL-4B column (1 x 43 cm)

Standard	MW	Retention Time
BSA	66,000	57 min
IgG	150,000	51 min
Apoferritin	443,000	44 min
hIgG aggregate	>20,000,000	22 min

Table 4.2 Determination of aggregate formation of native and modified hIgG by Sepharose CL-4B gel chromatography

Compounds	n	Mean % of aggregate form[a]
Native hIgG	4	6.5 (2.0)[b]
Oxidized hIgG	4	3.6[c] (0.8)
PDTA-hIgG	4	4.4[c] (0.6)
Sialic acid-hIgG	3	3.5[c] (0.9)

a: Different batches of hIgG were used

b: S.D. in parentheses

c: Modified antibodies were previously separated from the reagents by a Sephadex G-50 column before application onto a Sepharose CL-4B column.

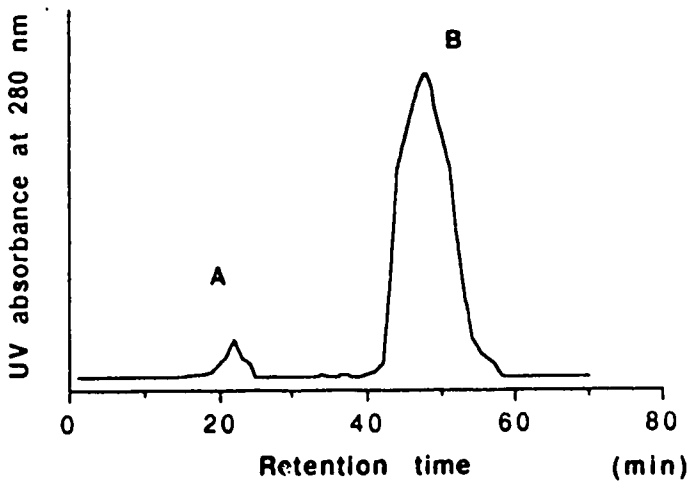


Fig. 4.3 Elution pattern of native hIgG solution by Sepharose CL-4B chromatography (peak A: aggregates, peak B: hIgG)

Similar experiments were also conducted to separate and determine the levels of aggregates by means of HPLC (TSK 3000SW column). At a flow rate of 1 mL/min good separation conditions were established. Fig. 4.4 shows the separation of native hIgG from aggregates present in the purchased bottle of reagent. Table 4.3 gives the results of aggregate detected after periodate oxidation and PDTA conjugation of the native hIgG assayed by HPLC.

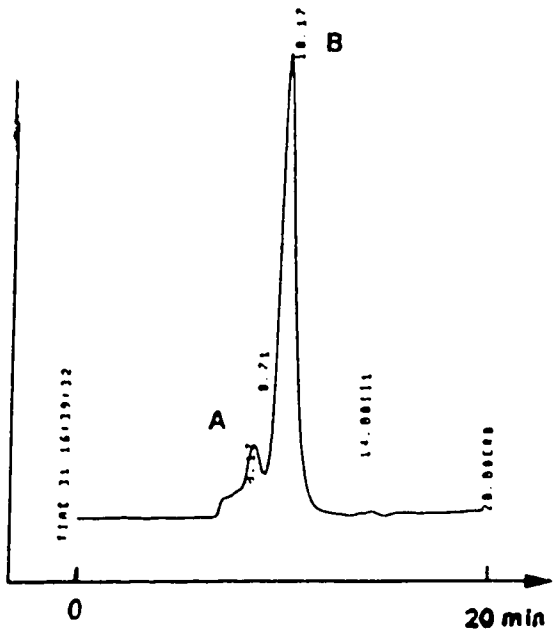


Fig. 4.4 Elution patterns of native hIgG by size exclusion HPLC (peak A: aggregates, peak B: hIgG)

Table 4.3 Determination of aggregate formation of hIgG, periodate-oxidized hIgG and PDTA-hIgG conjugate by HPLC

Antibodies	% aggregates[a]
Native hIgG	12.1 (9.9-14.2)
Oxidized hIgG	9.6[b] (9.5-9.6)
PDTA-hIgG	9.3[b] (8.9-9.6)

a: Percentage was determined by a SP4100 integrator (Spectra-Physics, CA, USA), n=2

b: Modified antibodies were previously separated by a Sephadex G-50 column before application onto HPLC.

4.4 Radioiodination

Since the first introduction of the method by Fraker and Speck in 1978, mild radioiodination of proteins with Iodogen as an oxidizing agent has been used extensively by many researchers. Because of the efficiency, simplicity and reproducibility (Saha, et al., 1989; Salacinski, et al., 1981; Boniface, 1986), the Iodogen method was selected in our study for the radioiodination of antibodies.

Radioiodination of native or modified antibodies at a level of 50 μg to 100 μg of protein with both ^{131}I and ^{125}I by the Iodogen method has produced consistent average radiolabeling yields of 59.8% (S.D. 4.24, n=28) throughout the study. Radiochemical purity after purification by a gel filtration column was 94.5% (S.D. 1.17, n=28) as determined by ITLC in 85% MeOH/H_2O (v/v). No attempt was made to determine the influence of radioiodination on the immunoreactivity of antibodies as preservation of the biological activity of this methodology has been verified by other researchers. (Wong, et al., 1988; Boniface, 1986).

4.5 Preparation and Biological Behaviour of GalNAc and GalGalNAc Antibody Conjugates *via* a Novel Fluorinated Linking Arm, HFGDE

Previous reports had shown that enzymatic treatment of glycoproteins to remove terminal sialic acids dramatically increased the blood clearance through specific hepatic receptors or liver lectins upon iv injection of these desialated glycoproteins (Morell, et al., 1968; Ashwell, et al., 1974). Rabbit, mouse and human hepatic receptors have been isolated with specificity for terminal Gal and GalNAc residues. The binding capacities of these receptors have also been tested *in vitro* (Bystrova, et al., 1985; Baenziger, et al., 1980; Sarkar, et al., 1979).

Instead of removing terminal sialic acid groups to expose the essential galactose residues, an analog, called neoglycoprotein (Stowell, et al., 1980), was prepared by the covalent attachment of desired sugar residues to proteins. Antibodies are natural glycoproteins and their structures can be altered by specific deletion and addition of sugar structures. In the present investigation, GalNAc and GalGalNAc were covalently attached to hIgG which served as a model antibody. A

novel fluorinated linking reagent, HFGDE, (Provided by Biomira Inc., Edmonton) was used for the conjugation. This technique, combined with the use of ^{19}F-NMR, provided an useful tool in the estimation of the number of sugar residues on antibody molecules.

4.5.1 Preparation of Amino Derivative of GalNAc

The synthetic scheme was given in section 3.2.1 (Fig. 3.1). A R_f value of 0.3 by TLC in solvent (1) was observed for compound 3 (Fig. 3.1), 5-phthalimidyl-α-D 2-aminoacetyl-2-deoxy-galactopyranoside. The spots were visualized both by a UV light for the conjugated aromatic system and by spraying with 5% H_2SO_4/EtOH (v/v) solution followed heating the TLC plate for the sugar portion. The ^{1}H-NMR spectrum of compound 3 (Fig. 3.1) shows chemical shifts, δ 7.7 (T, 2H), δ 7.9 (T, 2H) and δ 4.8 (S, 1H) for H^1 (at C-1), indicating an α-isomer structure (Appendix 1). Compound 3 (Fig. 3.1) was isolated in the pure form from a silica gel column in about 10% yield. It was then treated with hydrazine hydrate to provide compound 4 (Fig. 3.1) in 50% yield which showed a positive colour reaction with ninhydrin, indicating the formation of a primary amine group. The ^{1}H-NMR spectrum (Appendix 2) reveals the loss of the aromatic ring.

4.5.2 Linking Reaction of HFGDE to Amino Derivative of GalNAc and GalGalNAc, 4-Phenylbutylamine and 4-Amino-1-butanol

Fluorinated linking arm, HFGDE 5 (Fig. 3.2) was obtained as a colourless liquid after distillation at 0.4 mm Hg.

The ^{19}F-NMR spectrum ($CDCl_3$, C_6F_6 as a standard) (Appendix 3) of the linkinag reagent 5 (Fig. 3.2) shows two signals, δ 43.5 (4F) and δ 37.9 (2F). The ^{1}H-NMR ($CDCl_3$) spectrum (90 MHz) gave a signal of δ 4.0 (S, 3H) for ^{1}H in CH_3-group.

Excess methyl ester 5 (Fig. 3.2) was added to prevent the formation of diester of carbohydrates and amine compounds. The monomethyl ester was used in

the conjugation of biological macromolecules.

Because of the instability of the ester bond in water, the linking products of GalNAc and GalGalNAc **7a**, **7b** (Fig. 3.2) were not further purified after evaporation of ethyl ether. However, indirect evidence of the linkage was obtained when the model compounds, **7c** and **7d** (Fig. 3.2) were synthesized under similar conditions. The ^1H signals, δ 4.0 (S, 3H) of methyl ester (CH_3O-) of compound **7c** and **7d** (Fig. 3.2) are shown in Appendices 4 and 5. Because of the lipophilic properties, compound **7c** and **7d** (Fig. 3.2) were purified by silica gel columns eluted with solvents CH_2Cl_2 and EtOAc respectively. Disubstitution products of **7c** and **7d** (Fig. 3.2) were also obtained under certain conditions. An additional spot was visualized on silica gel TLC plates in those cases. And an additional peak was collected from silica gel columns as well. At the level of 20 to 40 μmole of starting amino compounds, under the conditions mentioned in section 3.2.4, monosubstitutes **7c** and **7d** (Fig. 3.2) were obtained in 45% to 65% yield after column purification. The following Table (Table 4.4) summarizes some of the properties of compounds **7c** and **7d** (Fig. 3.2) and their disubstitutes. ^{19}F-NMR spectrum of compound **7d** (Fig. 3.2) is given in Appendix 6.

Table 4.4 Some properties of linking products of HFDGE

Chemical[a] Structure	TLC R_f	^{1}H-NMR ppm	^{19}F-NMR ppm	Physical State
CH_3O-R-OCH_3 (5, Fig. 3.2)	0.90[b]	4.0[d] (S,3H)	43.5(4F) 37.9(2F)	Liquid
$\Phi(CH_2)_4NH$-R-OCH_3 (7c, Fig. 3.2)	0.60[b]	4.0[d] (S,3H)	43.4(2F) 42.0(2F) 37.2(2F)	Liquid
$\Phi(CH_2)_4NH$-R-$NH(CH_2)_4\Phi$	0.22[b]	NO 4ppm	ND	Solid
$OH(CH_2)_4NH$-R-OCH_3 7d (7d, Fig. 3.2)	0.62[c]	4.0[e] (S,3H)	43.4(2F) 42.6(2F) 37.2(2F)	Liquid
$OH(CH_2)_4NH$-R-$NH(CH_2)_4OH$	0.21[c]	ND	ND	ND

a: -R- : $-CO(CF_2)_3CO-$

b: in CH_2Cl_2

c: in EtOAc. Compound 5 had an Rf of 0.92 in this system

d: $CDCl_3$

e: $CDCl_3$ and DMSO-6d

ND: Not Done

Because of the symmetry of HFGDE, two signals from six fluorine atoms were observed in the ^{19}F-NMR spectrum. ^{19}F signals of 4 fluorine atoms, F1, F2, F5 and F6 (on C-3 and C-5) which are close to the carbonyl groups, are shifted down field (δ 43.5), while two fluorine atoms on C-2 have a chemical shift at a higher field (δ 37.9). The observed difference in chemical shifts between two ^{19}F signals of HFGDE is 5.6 ppm. In the case of monosubstitution in which the molecule becomes asymmetric, the 4 fluorine atoms on C-3 and C-5 are further divided into two groups that give two ^{19}F signals as can be seen in Table 4.4.

4.5.3 Conjugation of GalNAc, GalGalNAc and Non-sugar Linking Product 7d (Fig. 3.2) to hIgG

4.5.3.1 Determination of the Sugar Residues

^{19}F-NMR spectra of the sugar-hIgG conjugates show signals at δ 45.60 (4F) and δ 38.8 (2F) compared with δ 43.5 (4F) and δ 37.9 (2H) of HFGDE for the corresponding atoms using C_6F_6 as a standard. A difference of 6.8 ppm was observed in the chemical shifts between two ^{19}F-NMR signals of the conjugates (Appendix 7), while this difference was 5.6 ppm for the linking arm, HFGDE, itself. These differences enabled us to distinguish the coupled and uncoupled fluorines from their respective ^{19}F-NMR spectra.

Determination of the sugar residues per hIgG molecule was achieved by adding a known amount of NaF as an internal standard to ^{19}F-NMR samples. The ^{19}F-NMR signal of NaF appeared between the two ^{19}F signals of the conjugate. The chemical shift of NaF changed with the change of the pH and the concentration of the protein solution, while the difference of the two ^{19}F signals of the sugar conjugates was around 6.8 ppm consistently. The amount of fluorine atoms in sugar conjugates was obtained by comparison of the integers of the two ^{19}F-NMR signals of the conjugates with those of NaF standard (for calculation, see section 3.2.7, equations (3.5) and (3.6)). Five to 23 GalNAc residues and 3 to 6 GalGalNAc residues were attached per hIgG molecule with different initial ratios of

carbohydrates to hIgG. These results proved that hexafluoroglutaric acid methyl ester is a useful tool as a linking arm in conjugation reactions. The quantitation of sugar residues in proteins by using ^{19}F-NMR signals has provided us with a convenient alternative to the widely used phenol-sulfuric acid method.

4.5.3.2 Conjugation of Sugars to hIgG by Using Fluorinated Linking Arm

Conjugation of sugars to hIgG was achieved by the formation of the amide bonds between monosubstituted methyl ester (compound **7a**, **7b** and **7d**, Fig. 3.2) and the available amino side chains of the lysine or arginine groups on the hIgG molecule. There are about 71 lysine and 35 arginine residues per IgG molecule. Thus, different amounts of sugar residues could be incorporated by controlling the starting ratio of the sugar derivatives to antibody as indicated in Table 4.5. When the starting ratio approached 100, protein precipitation was observed which was probably due to high incorporation of the sugars *via* the fluorinated linking arm.

The hIgG-R-NH-butanol conjugate was used as one of the controls to study the possible influence of the conjugation process and the fluorinated linking arm itself on the biological distribution of the conjugates *in vivo*.

Table 4.5 Incorporation of the sugars on hIgG

Conjugates	Starting ratio (Sugar/hIgG)	Conjugation ratio[a] (Sugar/hIgG)
hIgG-R-GalNAc[b]	16	4
	30	6
	32	10
	62	23
hIgG-R-GalGalNAc[b]	20	5
	30	4
hIgG-R-NH-butanol[b]	50	5
	80	11

a: Amount of sugar residues was determined by ^{19}F-NMR signals.

b: R: -CO$(CF_2)_3$CO-

4.5.4 Stability Study of the Ester Bond in HFGDE

The question has arisen about the stability of the ester bond in the fluorinated linking arm and the amide bond formed after the conjugation because of the extremely high electronegativity of the fluorine atoms in the molecule. A stability study of the amide bond formed between the linking arm and the HSA molecule showed that the conjugate was stable after storage at 4°C for 14 days (Koganty, unpublished data). The stability study of the ester bond of a monosubstituted methyl ester with PAB-PDTA 7e (Fig. 3.2) was investigated. PAB-

PDTA was chosen because of its potential use as a metal chelate and easy of quantitation by means of ^{1}H-NMR spectrophotometry with the aromatic protons as a standard to calculate the decrease of CH_3- proton signals caused by hydrolysis of the methyl ester bond.

4.5.4.1 Linking Reaction of PAB-PDTA

The ^{19}F-NMR spectrum of the linking product, compound 7e (Fig. 3.2) shows three ^{19}F signals, 37.24 (2F). 44.54 (2F) and 46.87 (2F) (Appendix 8) compared with two signals of linking reagent itself, 37.88 (2F) and 43.49 (4F). Asymmetric linking of PDTA to linking arm (compound 5, Fig. 3.2) caused fluorine signals split into three groups. The presence of CH_3- proton signals, δ 3.9 (S, 3H) in ^{1}H-NMR spectrum (Appendix 9) indicated the preservation of one methyl ester in compound 7e (Fig. 3.2, monosubstitution).

4.5.4.2 Study of Hydrolysis of the Methyl Ester Bond in Compound 7e (Fig. 3.2)

A semilog plot in Fig. 4.5 gives a half life of 2.1 hours for hydrolysis of methyl ester bond at 20^{0}C as measured by ^{1}H-NMR. Because of the survival of the ester bond, formation of the amide bond between it and the amino group on proteins became possible. The amide bond formed in this reaction was much more stable than ester bond ($T_{1/2}$ = 14 days at 4^{0}C for amide bond, from Dr. Koganty's unpublished data). On the other hand, the presence of the free linking arm resulting from inadequate separation post reaction would induce dimer formation because of the survival of the ester bond.

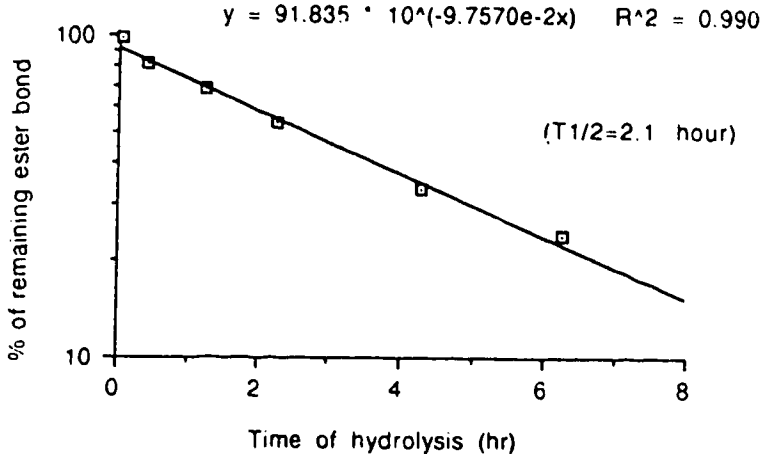


Fig. 4.5 Hydrolysis of ester bond of compound 7e (Fig. 3.2) at 20°C

The successful conjugation of GalNAc, GalGalNAc to hIgG and MAb 155H.7 and the convenience in estimating sugar residues by ^{19}F-NMR signals demonstrated that the fluorinated linking arm provided a useful tool in selectively modifying antibodies.

4.5.5 Biodistribution of Sugar Conjugates in Normal Mice

The sugar-hIgG conjugates with about 6 and 23 GalNAc and 4 GalGalNAc residues per hIgG molecule were used in the tissue biodistribution studies in normal male ICR mice. The aggregates of the sugar conjugate preparations were about 10% as evaluated by a Sepharose CL-4B column (1 x 43 cm), and about 5% aggregates as determined for a non-sugar conjugate, hIgG-R-NH-butanol 8d (Fig. 3.2), which was at the same level as that of native hIgG (different amount of aggregates were found in different batches of hIgG).

Because of the importance of the hepatic receptor for sugar-conjugate accumulation, liver uptake and blood clearance were of the most interest in the design of the tissue distribution studies. Alveolar macrophages were also reported to show a receptor-mediated binding to some glycoproteins. Thus the tissues of

interest were dissected and the % injected dose was calculated to determine the changes upon the attachment of GalNAc and GalGalNAc onto hIgG. The observed data are shown in Table 4.6 and Fig. 4.6.

The *in vitro* inhibitory binding studies of asialoglycoproteins to human and rabbit hepatic receptors have indicated that glycoproteins with terminal GalNAc residues exhibited strong inhibitory ability to the binding (Sarkar, et al., 1979; Baenziger, et al., 1980). The results from our current investigation provided an *in vivo* evidence for the existence of the hepatic receptor for the modified hIgG with GalNAc termini in which the GalNAc conjugate was transferred rapidly from the circulation into the liver. The most significant differences ($p < 0.01$) were found in the liver uptake and blood level at 15 minutes PI between native hIgG and GalNAc conjugated hIgG (Fig. 4.6). Similar results in terms of liver uptake and blood level ($p < 0.01$) were also observed between hIgG-GalGalNAc conjugate and native hIgG control (Fig. 4.6).

The presence of dehalogenation enzymes in liver tissues will always present problems when interpreting data resulting after the *in vivo* administration of radiohalogenated proteins. Our observation of high levels of liver radioactivity in animals receiving neoglycosylated antibodies at an early period after iv injection (15 minutes) indicated one of two possibilities: (a) that the dehalogenation of native hIgG is faster than the neoglycosylated form or (b) that the neoglycosylated protein localize in the liver at an extremely high rate, thus explaining the high level of radioactivity at that time period. The second possibility gains evidence when one examines the published literature (Ashwell, et al., 1974, 1982). The observation of the difference in liver levels of radioactivity in animals injected with either glycosylated or native hIgG after 1 hour PI indicates an extremely rapid rate of dehalogenation of the neoglycosylated antibodies by the liver hepatocyte. This is consistent with the observation of Gore, et al., (1989) who have shown a considerable amount of dehalogenation of glycosylated albumin after a single pass in a perfused rat liver. Thus, one might conclude from our observation that the rate of liver uptake of neoglycosylated antibodies is higher than the rate of its

Table 4.6 Effect of sugar conjugation to hIgG on tissue biodistribution

Time (hr)	Tissues	hIgG-GalNAc[a]	hIgG-GalGalNAc[b]	Native hIgG
0.25	Blood	13.5 (1.51)[c]	9.06 (0.21)	29.3 (1.07)
	Liver	21.2 (2.12)	21.8 (3.84)	8.12 (0.15)
	Lungs	10.8 (1.52)	14.2 (3.46)	9.85 (0.44)
	Spleen	9.29 (1.45)	9.71 (1.35)	4.85 (0.25)
	Kidneys	5.26 (0.32)	4.65 (0.43)	7.19 (0.68)
1	Blood	9.74 (0.47)	5.27 (0.16)	24.2 (2.18)
	Liver	8.70 (1.04)	9.73 (1.59)	7.15 (1.74)
	Lungs	4.91 (0.83)	6.71 (2.55)	6.92 (1.30)
	Spleen	6.02 (1.38)	5.03 (0.41)	4.14 (0.43)
	Kidneys	4.63 (0.84)	3.61 (0.13)	5.83 (0.91)
6	Blood	3.57 (0.33)	2.07 (0.29)	16.8 (0.92)
	Liver	3.33 (0.43)	4.13 (0.77)	3.82 (0.16)
	Lungs	1.79 (0.35)	1.47 (0.51)	6.18 (1.24)
	Spleen	2.20 (0.97)	1.84 (0.23)	3.03 (0.13)
	Kidneys	2.01 (0.42)	1.82 (0.27)	4.16 (0.64)
12	Blood	1.96 (0.15)	ND	14.5 (1.22)
	Liver	1.76 (0.18)	ND	3.15 (0.40)
	Lungs	1.89 (0.44)	ND	4.17 (0.89)
	Spleen	1.51 (0.31)	ND	1.90 (0.26)
	Kidneys	2.12 (0.85)	ND	3.28 (0.34)

a: With 6 sugar residues

b: With 4 sugar residues

c: S.D. in parentheses, n=3-4 aminals injected iv with conjugates

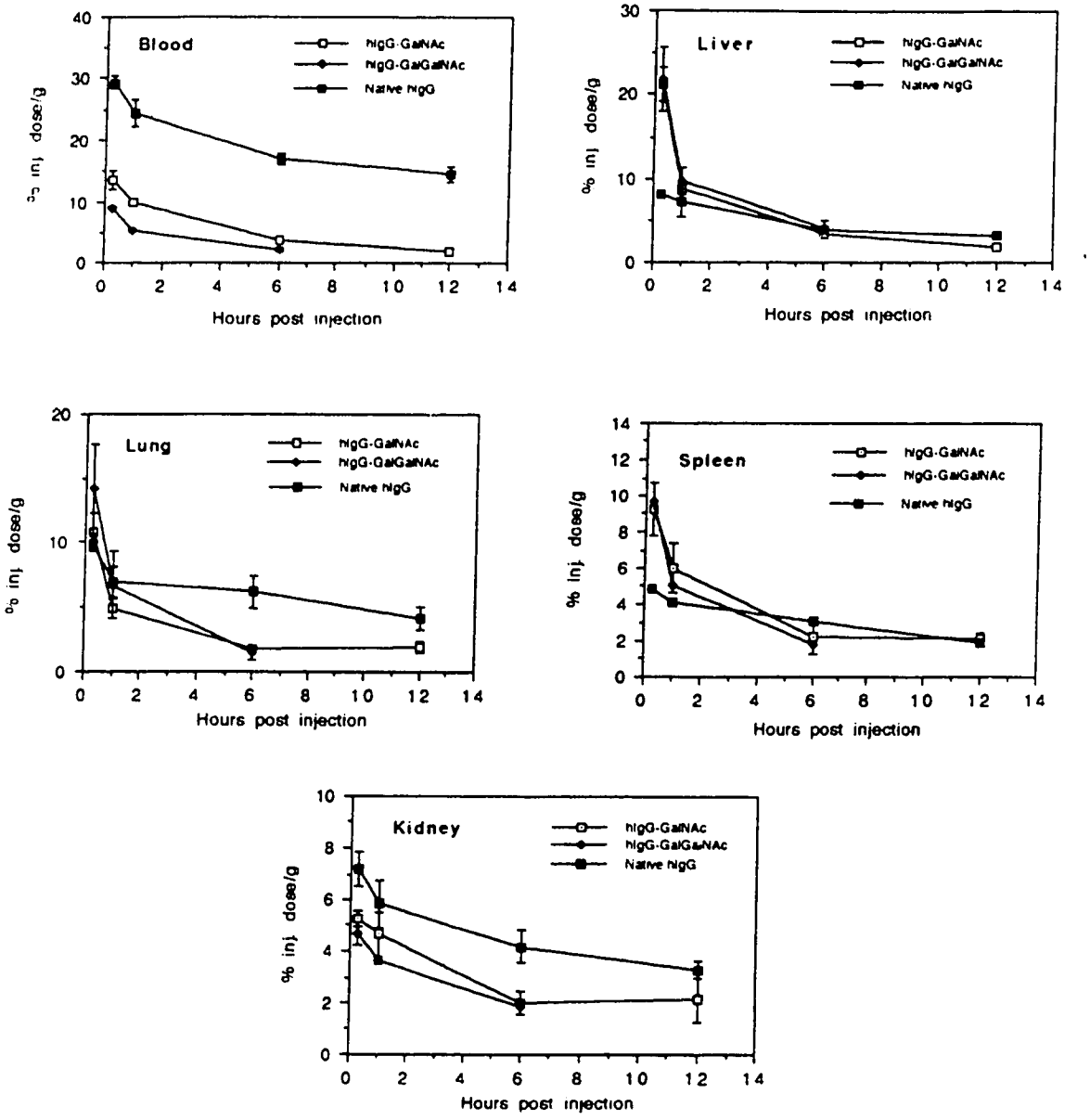


Fig. 4.6 Time course of selected tissue distribution of sugar conjugates and native hIgG (6 residues for hIgG-GalNAc and 4 residues for hIgG-GalGalNAc) in normal ICR mice.

dehylogenation by this organ, and that these same antibodies have a much higher liver uptake when compared to their native form at an early time period.

Increased spleen uptake was observed at an early time point (15 minutes PI) for both sugar conjugates while returning to normal levels after 1 hour PI. Either the formation of a denatured antibody during the manipulation process or a similar mechanism to the liver uptake may be attributed to the observed increase in the spleen. Since this phenomenon was not observed for a control experiment in which a non-sugar compound was conjugated to hIgG under the same conditions as those for the conjugation of the sugars, and the same protocol was used in the biodistribution study, it seems that the uptake in spleen was induced by some kind of sugar receptor mechanism although the direct evidence is lacking.

Kidney uptake of both sugar conjugates was diminished in the investigated period as compared to native hIgG (15 minutes to 12 hours, Fig. 4.6). No early effect on lung uptake but a decrease in lung radioactivity after 1 hour PI was observed (Fig. 4.6). Similarities in declining rates of plasma radioactivity with renal and lung radioactivity at the correspondence time periods would suggest that the decreased kidney and lung level mainly reflects the decreased blood level in the case of the sugar conjugates.

Although it was reported that the *in vitro* inhibitory ability of the binding of asialoglycoproteins to the hepatic receptor was considerably greater for the terminal, non-reducing GalNAc glycoproteins rather than terminal galactosylated glycoproteins (Baenziger, et al., 1980; Sarkar, et al., 1979), there is no *in vivo* evidence for this to our knowledge. An interesting finding in this investigation was that a similar pattern of liver uptake of GalGalNAc conjugate (Gal terminus, 4 residues) as that of GalNAc conjugates (6 residues) was observed (21.25%, 8.70% and 3.33% *vs* 21.83%, 9.73% and 4.13% at 0.25, 1, 6 hours PI), whereas the blood level of GalGalNAc-hIgG conjugate was lower than that of GalNAc-hIgG conjugates in the investigated time period (up to 6 hours) (Fig. 4.6).

In vivo investigation on binding and endocytosis of asialo-glycoproteins revealed the complexity of this process. Injection of small amounts (< 1 μg/ 100 g

body weight) of asialotransferrin was reported to result in an equilibrium between the plasma and the liver receptor and was not followed by a signal for endocytosis. On the other hand, a higher dose (up to 509 μg/ 100 g body weight) resulted in lower blood level and increased catabolism of the asialoglycoprotein (Regoeczi, et al., 1978; 1979; Tolleshaug, et al., 1981). It was also reported that glycopeptides differing in affinities for the isolated receptor by as much as 750-fold, exhibited similar kinetics of endocytosis by isolated hepatocytes (Baenziger, et al, 1980). These authors also proposed that only those glycoproteins which had the correct spacing of their terminal Gal or GalNAc residues could induce conformational changes in the receptor molecule and probably triggered endocytosis. Their investigation results also showed that the glycopeptides bearing 4 to 5 O-glycosidically linked GalGalNAc or GalNAc moieties were found to be endocytosed by the isolated hepatocytes with the same kinetics as asialo-orosomucoid; whereas glycopeptides which bore only two O-glycosidically linked moieties were not subject to endocytosis by hepatocytes.

Although GalNAc conjugates have a higher *in vitro* affinity for the liver receptors, we have failed to observe a faster elimination when compared to GalGalNAc-conjugated hIgG. This supports the concept that the binding to the receptor was necessary but not sufficient to elicit the endocytosis. From the investigation of the recycling mechanism of the hepatic receptor (Tanabe, et al., 1979), it was proposed that ligands appeared to enter the cell bound to the receptor but intracellular separation of the ligand from the receptor preceded ligand destruction. From this point of view, strong binding of GalNAc conjugate to the receptor became a negative influence on the separation process at this stage of endocytosis, although a more complicated process may be involved. The higher blood levels observed for the GalNAc conjugates (6 residues), when compared to these of the GalGalNAc conjugates (4 residues) suggest less catabolism for the GalNAc conjugate. No attempt was made to explore the nature of the difference in the handling of the two sugar conjugates of hIgG by murine liver tissues.

A study of the effect of increased GalNAc residues on tissue biodistribution (6 to 23 on hIgG molecule) was also conducted (Fig. 4.7). A quantitative difference

was seen in blood clearance between hIgG-GalNAc-1 (6 residues) and hIgG-GalNAc-2 (23 residues) at 0.25 and 1 hour periods (13.51%, 9.74% vs 10.50%, 6.80% respectively). Since liver was the major organ of sugar conjugate uptake and metabolism, the peak uptake might be expected to occur at an earlier stage for the high-incorporation conjugate. Quantitative differences occurring in blood levels while no significant differences being observed in liver uptake at 15 minutes PI allows us to postulate that the peak liver uptake was probably less than 15 minutes (the earliest data available in this experiment was 15 minutes) since the liver was the major clearance organ. The increased lung radioactivity was also observed at 15 minutes and 1 hour PI. The accelerated catabolism of the sugar conjugates in lungs might partly contribute to the blood clearance of the high-incorporation sugar conjugate as well.

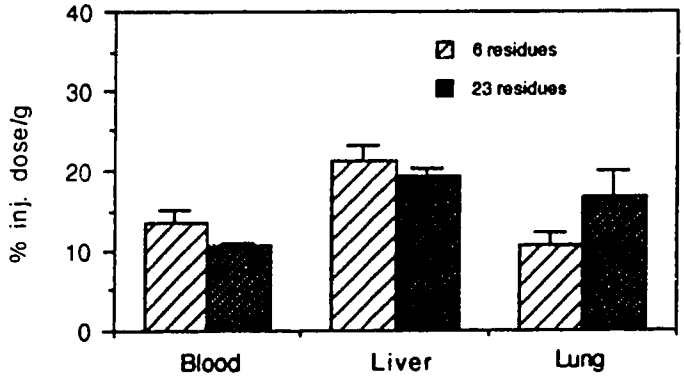


Fig. 4.7 Effect of increased GalNAc residues on tissue biodistribution in normal mice 15 minutes PI

There was no significant difference observed in tissue biodistribution in mice between the two controls (Fig. 4.8). Normal tissue distribution was observed for non-sugar conjugate **8d** (Fig. 3.2, 5 residues per antibody molecule) when the same reaction conditions as those for the conjugation of the sugars were used indicating validity of the conjugation method. Thus the observed increase in liver uptake was

induced by conjugated sugars, and not the linking arm itself. The sugar conjugation technique by using HFGDE as a linking reagent can be applied to MAbs to conjugate desired reagents to selectively modify their biological behaviour *in vivo*.

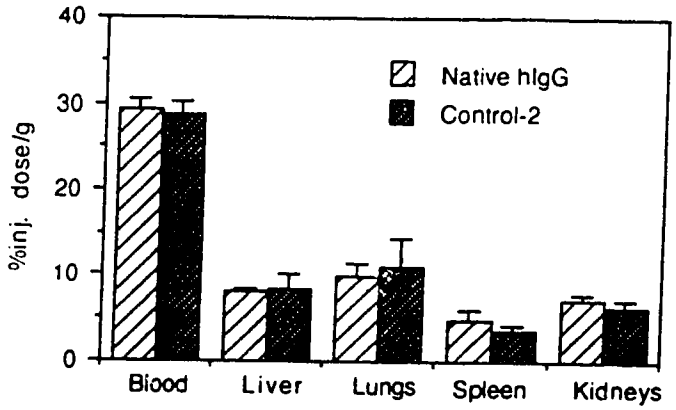


Fig. 4.8 Comparison of tissue distributions of the two controls. Control-2 was the amino-butanol conjugated hIgG 15 minutes PI

4.6 Sialic Acid-hIgG Conjugate

4.6.1 Preparation of Sialic Acid-hIgG Conjugate

Oxidative cleavage of C=C double bond in compound **9** (Fig. 3.3) was indicated by the disappearance of δ 5.9 (M, 1H), δ 5.2 (D, 1H) and δ 5.05 (D, 1H) in ^{1}H-NMR (D_2O) spectrum (Appendix 10). The existence of -CHO in compound **10** (aldehyde derivative of **9**, Fig. 3.3) has been characterized by chemical shift as δ 8.3 (S, 1H) in ^{1}H-NMR spectrum.

A standard curve of resorcinol assay with a correlation coefficient (r) of 0.99 is shown in Appendix 11.

The data in Table 4.7 shows that sialic acid residues increase with an increase in reaction time. Fifteen residues per hIgG molecule were attached after a 60 hours reaction time. There was no sialic acid coupled onto native hIgG molecules under control conditions, indicating the validity of the method.

Table 4.7 Sialic acid residues incorporated per hIgG molecule

Starting ratio (Sialic acid/hIgG)	Reaction time (h)	Residues/hIgG
100:1	6	3
100:1	40	8
100:1	66	15
Conjugation control[a]	15	0

a: Native sialic acid, instead of aldehyde derivative of sialic acid, was used in the control test.

4.6.2 Biodistribution in Male ICR Normal Mice

A preparation of sialic acid-hIgG conjugate containing about 8 residues per hIgG molecule and less than 10% aggregates, as determined by a Sepharose CL-4B column, was used in the animal biodistribution study.

Two set of experiments were carried out. In the case of single tracer test, native hIgG and sialic acid conjugates were both labeled with ^{125}I and injected into a separate set of mice. In paired labeling experiments native hIgG was labeled with ^{125}I while the sialic acid-hIgG conjugate was labeled isotopically with ^{131}I. These were then mixed and injected simultaneously *via* the tail vein into mice. The advantage of the paired labeling experiment is that variations among individual mice can be eliminated since each experimental animal serves as its own control (Pressman, et al., 1957).

The results from the single tracer experiment are given in Table 4.8. It appeared that there were no significant differences observed in liver uptake and blood clearance between radioiodinated sialic acid-hIgG conjugate and native hIgG.

Table 4.9 shows the data of a double tracer experiment. Similar results as those observed in the case of single tracer experiments were obtained.

Table 4.8 Percent injected dose/g tissue in single label experiment

Conjugates	Time	Tissues[a]				
	(hr)	Blood	Liver	Lungs	Spleen	Kidneys
Sialic acid[b] Conjugate	0.25	27.82 (1.31)	12.21 (2.11)	8.93 (2.42)	5.45 (0.59)	6.19 (0.32)
Native hIgG	0.25	28.45 (1.90)	9.81 (0.96)	13.40 (2.30)	6.51 (0.80)	7.33 (1.30)
Sialic acid Conjugate	1	16.89 (2.25)	7.98 (1.16)	5.41 (0.57)	3.82 (0.90)	5.57 (0.99)
Native hIgG	1	18.82 (1.87)	7.15 (1.20)	6.14 (1.76)	4.23 (0.78)	5.83 (0.09)
Sialic acid Conjugate	6	13.78 (1.23)	4.12 (0.52)	3.49 (0.40)	1.55 (0.30)	2.89 (0.39)
Native hIgG	6	15.23 (0.97)	3.99 (0.23)	5.13 (1.12)	2.96 (0.43)	3.79 (0.62)

a: n=3-4, S.D. in parentheses

b: Eight sialic acid residues per hIgG molecule

Table 4.9 Percent injected dose/g tissue in paired labeling experiment

Conjugates	Time	Tissues[a]				
	(hr)	Blood	Liver	Lungs	Spleen	Kidneys
Sialic acid Conjugate	0.25	26.87 (2.78)	11.78 (1.98)	11.23 (1.20)	6.72 (0.61)	5.33 (0.21)
Native hIgG	0.25	27.96 (2.07)	10.34 (3.8)	12.01 (1.72)	6.47 (1.26)	6.44 (0.79)
Sialic acid Conjugate	9	9.58 (0.91)	2.56 (0.33)	3.44 (0.40)	1.59 (0.16)	3.06 (0.41)
Native hIgG	9	8.97 (1.43)	2.67 (0.56)	2.51 (0.67)	2.13 (0.18)	2.98 (0.22)

a: S.D. in parentheses, 15 minutes PI with 2-4 mice per time point

The anti-recognition effect of sialic acid in the liver uptake of glycoproteins was first described by Morell, et al., (1968). It was observed that intravenously injected radiolabeled asialo-ceruloplasmin was taken up by the parenchyma cells of the rabbit liver and cleared from blood rapidly. The Gal group exposed after removal of the terminal sialic acid was shown to be responsible for the clearance *via* a specific liver receptor. The protecting effect of sialic acid was further verified by enzymic replacement of the missing sialic acid of the desialylated glycoprotein (Hickman, and Ashwell, 1970). Interestly, our experiments indicated that the conjugation of sialic acid to the amino acid backbone had no apparent effect on

biodistribution of hIgG in normal mice.

One of the explanations for the role of sialic acid played in the liver uptake was the presence of negative charges on the molecule. It was discovered that a negatively charged group at C-6 of galactose interfered with the *in vitro* binding of the neoglycoprotein to the hepatic lectin, and a conclusion was drawn that the lectin possessed at least one negatively charged group at the binding site. This was responsible for the inability of the sialated native glycoprotein to bind to the mammalian hepatic lectin (Lee, R.T., 1982b). The existence of sialic acid on liver receptors was also demonstrated by characterization of separated liver lectin (Kawasaki and Ashwell, 1976b; Tanabe, et al., 1979). However, the increase in negative charges as the result of more incorporation of sialic acid on the amino acid backbone of hIgG molecule did not induce further reduction in liver uptake as observed in our experiment. This might be due to the fact that the incorporation of the sialic acid did not occur on the carbohydrate portion of antibodies and did not mask any exposed galactose residues on hIgG molecule. This also implied that the uptake of antibodies by the liver is obviously controlled by more than one mechanism which should be the object of further research.

In the case of splenic uptake of either native hIgG or the sialated hIgG, it appeared that the level of spleen uptake for the sialated form was decreased as a function of time, although the initial uptake of the two forms of hIgG did not show any significant difference. This may be due to either an increased splenic catabolism of the sialated hIgG or an increase with the activity of the dehalogenation enzyme as a result of increasing of the negative charge on the antibody molecule.

4.7 Effect of the Structural Modifications of the Carbohydrate Moiety of hIgG and Monoclonal Antibodies on the Biological Distribution in Mice

The oligosaccharide moieties of hIgG and MAb 155H.7 were modified in three different ways: (1) oxidization by sodium periodate followed by reduction with sodium cyanoborohydride ($NaCNBH_3$) which resulted in the formation of an alcohol group; (2) oxidized antibodies were conjugated with tyrosine hydrazide (TH) which

resulted in the addition of an amino acid; and (3) oxidized antibodies were conjugated with DNPH which resulted in the addition of a lipophilic group.

The conjugated antibodies were evaluated for possible aggregate formation by a Sepharose CL-4B column as mentioned before. There were no apparent increases in aggregate formation when compared to the native antibodies.

DNPH-conjugated MAb 155H.7 was evaluated for its immunoreactivity by ELISA assay with HSA-Tα as the antigen. Two batches of DNPH-MAb 155H.7 were tested (Fig. 4.9). The immunoreactivity of the antibody conjugate declined as a result of conjugation of DNPH. Because of the lipophilic property of DNPH, the later was dissolved in DMSO before addition to the oxidized antibody solution. Addition of a few microliters of DMSO solution was directed to the surface of the antibody solution to avoid precipitation which might contribute to the reduction of the immunoreactivity detected by ELISA assay.

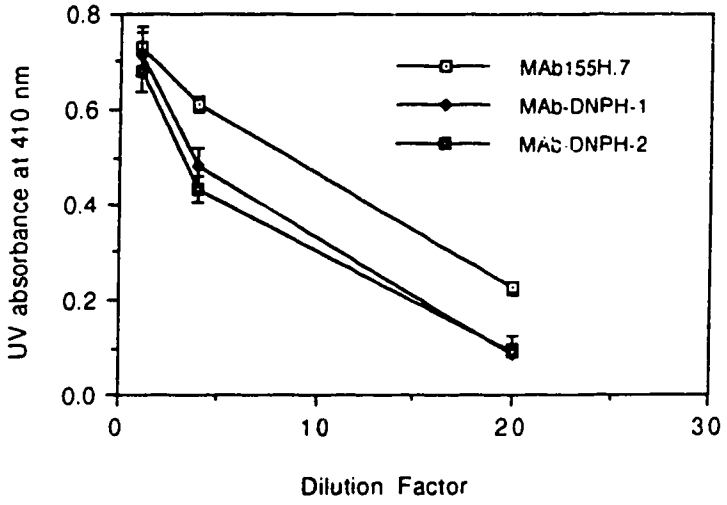


Fig. 4.9 ELISA assay of DNPH-MAb 155H.7 conjugate

The biological distribution data of modified antibodies (hIgG and MAb 155H.7) labeled with ^{125}I in normal ICR mice are presented in Table 4.10. Similar blood level and liver, lung and kidney uptake were observed for all modified antibodies except that higher spleen uptake was observed for DNPH conjugated MAb 155H.7. This might be the consequence of the presence of a small amount of denatured antibody during the manipulation process. Fig. 4.10 shows the comparison of the blood level and liver uptake of the modified antibodies and native antibody in normal mice 15 minutes PI. Modification of oligosaccharide moiety of antibodies by periodate treatment destroyed terminal reducing sugars and some of the innerchain neutral sugars. It can be concluded from our experiments that modification of the carbohydrate chains in the Fc portion of the hIgG and MAb 155H.7 by the above treatment did not have any apparent influence on their blood level and liver uptake, at least at an early stage after iv injection. Thus the replacement of the terminal carbohydrate group by either an alcoholic formation, or an amino acid substitution, or even addition of a lipophilic functionality did not affect the antibody uptake by the liver or its clearance from the circulating blood compartment.

It is known that the carbohydrate-specific receptors in the liver are responsible for the clearance of many desialated glycoproteins from the circulation *via* the hepatic pathway. Chemical conjugation of Gal (Mattes, et al., 1987), GalGalNAc and GalNAc (this study) to hIgG molecules did induce rapid liver uptake and fast clearance from the circulation upon iv injection into mice. It was reported that the presence of a terminal sialic acid in carbohydrate chains of IgG molecules protected the Gal residues from recognition by the carbohydrate-specific receptor on hepatocytes and this was accounted for the survival of IgG molecules in the circulation (Winkelhake, et al., 1976). However, the effect of the presence of galactose residues of the carbohydrate chain on the catabolism rate of IgG which has only about half of the carbohydrate chains having sialic acid residues and others having exposed Gal (Mizuochi, et al., 1982), is somewhat controversial. Nose and Wigzell (1983) reported that when they used MAbs (IgG_{2b}) which were depleted

Table 4.10 Comparison of biodistribution of ^{125}I-labeled modified antibodies in normal ICR mice 15 minutes PI

Conjugates	Tissues (% inj. dose/g)[a]				
	Blood	Liver	Lungs	Spleen	Kidneys
hIgG-OH[b]	25.81	8.21	13.10	5.43	6.34
	(2.93)	(1.92)	(2.55)	(1.51)	(0.93)
hIgG-TH[c]	26.80	8.01	11.60	4.59	6.01
	(1.91)	(0.92)	(1.64)	(0.76)	(0.63)
MAb-OH[b]	27.30	9.06	10.40	4.88	6.15
	(2.47)	(1.48)	(2.36)	(0.28)	(0.41)
MAb-TH[c]	26.80	8.81	ND	ND	ND
	(2.60)	(1.31)			
MAb-DNPH[d]	25.90	9.25	9.09	8.41	6.22
	(2.65)	(0.57)	(1.60)	(1.23)	(1.25)
Native hIgG	28.20	9.11	9.90	4.85	7.12
	(1.20)	(0.50)	(0.42)	(0.10)	(0.67)
Native MAb	31.00	8.27	13.80	4.38	8.51
	(2.30)	(0.55)	(2.73)	(0.44)	(1.04)

a: S.D. in parentheses

b: formation of an alcohol group

c: addition of tyrosine hydrazide

d: addition of about 6 to 7 DNPH residues
n=3-5

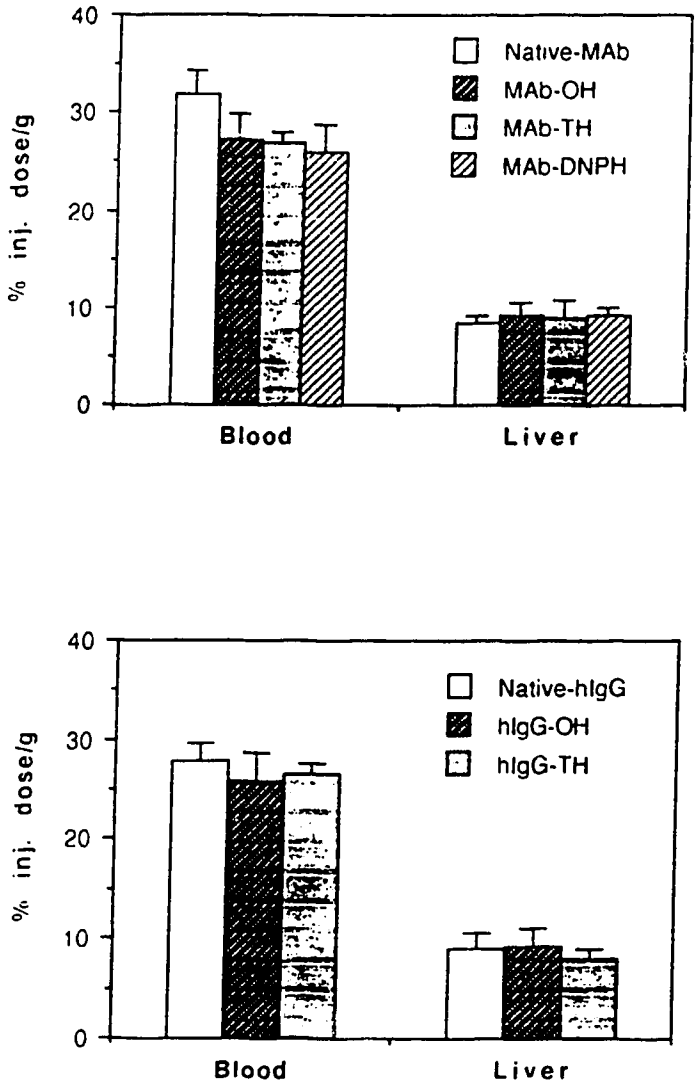


Fig. 4.10 Comparison of blood level and liver uptake of antibodies after modification of their oligosaccharide moieties at 15 minutes PI

from their asparagine-linked carbohydrate chains, these MAbs were eliminated almost in the same manner as control Ig molecules. Tao and Morrison, (1989) indicated that aglycosylated chimeric mouse-human IgG_1 was found to have the same half-life range as that reported for mouse MAb of IgG_1, IgG_{2a} and IgG_3. The question arises that if indeed IgG is removed from the circulation by the asialoglycoprotein receptor, then aglycosylated IgG and the IgG with their terminal sugars chemically destroyed should have longer half-lives and lower liver uptake. Our results therefore suggested that the asialoglycoprotein receptor may not play an important role in the clearance of IgG from the circulation in mice.

4.8 Periodate Oxidation of Antibodies

The periodate oxidation of carbohydrates was reviewed extensively by Bobbitt (1956). The rationale for using carbohydrate moieties as the modification site on antibodies is based on the fact that they are located in the Fc region of the heavy chains, and that they are not frequently involved in the antibody-antigen binding activity. Generally, according to current literature (O'Shannessy, et al., 1987; Murayama, et al., 1978; Bobbitt, 1956), periodate oxidation is performed in acetate buffer, at pH 5.2, at room temperature (25°C-28°C) or at 4°C for 30 minutes in the dark.

4.8.1 Study of the Stability of Oxidized hIgG during storage

For the purpose of our studies it was of importance to determine the stability of oxidized IgG prior to further conjugation with any chelate. In our present work it was found that the number of DNPH residues coupled to IgG molecules declined to 3-4 after 10 hours of lyophilization of the oxidized hIgG when compared to 8-9 residues while the conjugation was performed immediately after separation of excess $NaIO_4$. No ^{111}In radioactivity was found in protein bound, as assayed by ITLC, when chelating agent PAB-PDTA was reacted with the oxidized hIgG which had been lyophilized for 15 hours. Table 4.11 shows the effect of lyophilization time on the conjugation activity of oxidized proteins.

Table 4.11 Effect of lyophilization on the conjugation activity of oxidized proteins

Proteins[a]	Lyophilization Time (hr)	DNPH residues Coupled
hIgG[b]	0	9.47 (0.38)[c]
	10	3.57 (0.12)
	15	0% protein bound[d]
HSA[e]	0	0.20 (0.11)
	9	0

a: An oxidized hIgG solution collected from a G-50 column was lyophilized

b: hIgG was oxidized by periodate at 28°C for 30 minutes. A DNPH/hIgG ratio of 180:1 was used and conjugation reaction was carried out at 4°C for 15 hours

c: S.D. in parentheses

d: PAB-PDTA was conjugated to oxidized hIgG which had been lyophilized after separation by a G-50 column, percentage of protein bound ^{111}In radioactivity was assayed by ITLC

e: HSA was used as a non-glycoprotein molecule when investigating possible periodate oxidation of the amino acids on proteins; the oxidation conditions were the same as those in (a)

Oxidized antibodies may lose aldehyde groups gradually by intra- or intermolecular condensation with available amino groups or other hydroxyl groups. Intramolecular condensation-induced cross linking was reported by Brow (1986). In our present experiments (Table 4.12) we found that there was no apparent increase in oligomer or dimer formation for oxidized antibodies stored at 4°C up to 15 days as evaluated by a Sepharose CL-4B column and HPLC (TSK 3000SW) (Fig. 4.11), indicating intermolecular condensation is unlikely. However, possible intramolecular condensation could not be detected by these methods.

Table 4.12 Aggregate formation in periodate-oxidized[a] antibodies during storage

Storage time (day)	% aggregate Formation	Method
1	2.9 (n=2)	CL-4B column
3	3.1 (n=1)	CL-4B column
5	3.0 (n=1)	CL-4B column
13	3.3 (n=1)	CL-4B column

a: Antibodies were oxidized by 30 mM of periodate at 28°C for 30 minutes. The oxidized antibodies were stored at 4°C

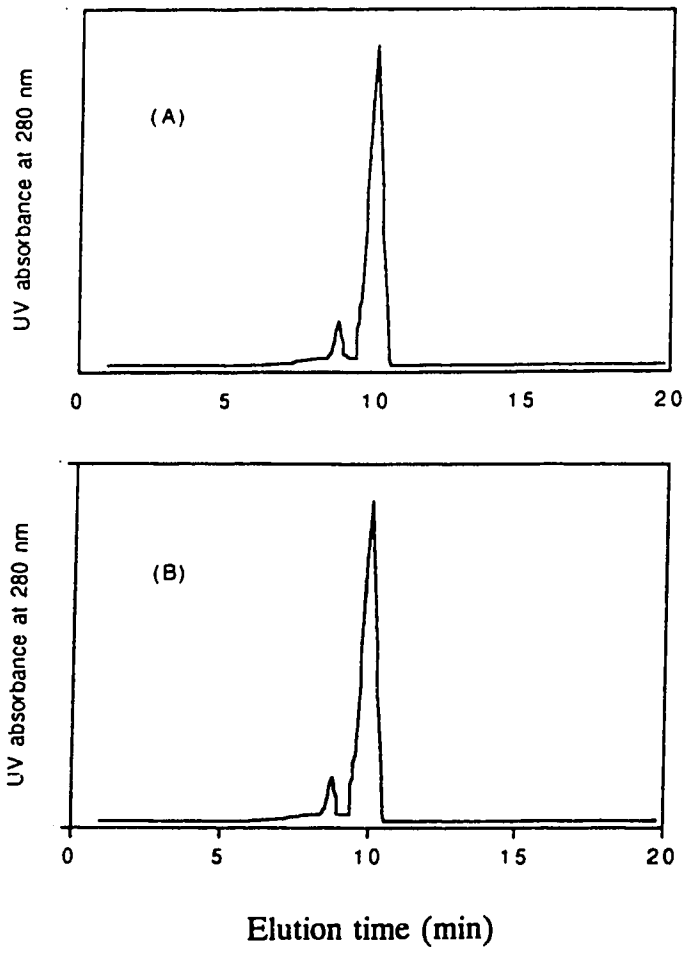


Fig. 4.11 Evaluation of oligomer formation by SE HPLC. A: oxidized hIgG was stored at 4°C for one day. B: same batch of oxidized hIgG was stored at 4°C for 15 days. All molecule are eluted within 20 minutes

4.8.2 Theoretical Prediction of Oxidative Sugars on Oligosaccharide Chains of IgG Molecule

Structures of the two oligosaccharide chains of human IgG molecule are given in Fig. 4.12. There are two oligosaccharide chains per IgG molecule. While the chain II with either one or two sialic acid terminal residues is only about 25 % of the total carbohydrate content, the rest is composed of chain I. The

oligosaccharide chains are located in CH2 portions of the two heavy chains of the IgG molecule. The conformation picture of one of the two oligosaccharide chains is expressed in Fig. 4.13 for the purpose of clarity in the estimation of the number of oxidative sites on the oligosaccharide. According to Rothfus and Smith (Rothfus and Smith, 1963), any terminal non-reducing monosaccharide, and pyranosidically-linked neutral sugars, which are not substituted at carbon 3 within oligosaccharide chains may be subject to periodate oxidation. Thus seven terminal non-reducing sugars, and six innerchain monosaccharides were counted as oxidative sugars in one hIgG molecule. However, some of them, especially those of innerchain monosaccharides, may not be oxidized under the conditions used in our experiments. Thus, in the case of monosaccharide or disaccharide conjugated proteins, the number of sugars vulnerable to periodate oxidation may be predicted more easily. The degree of oxidation was estimated from the number of DNPH residues associated with the antibody molecule after periodate oxidation.

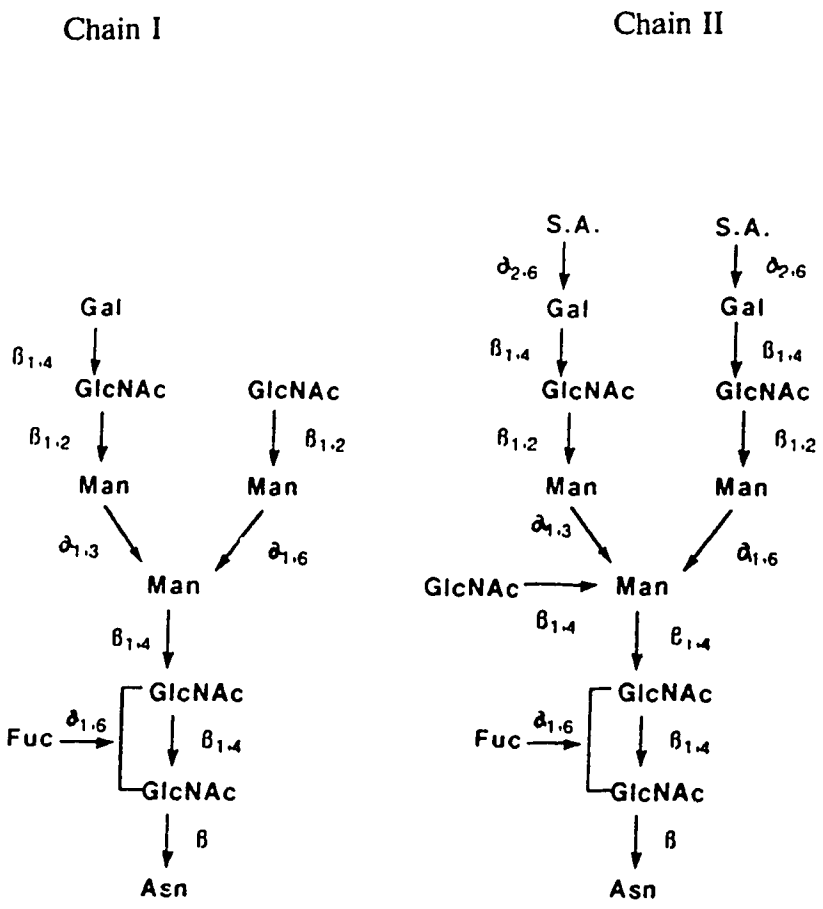


Fig. 4.12 Oligosaccharide chains of a hIgG molecule

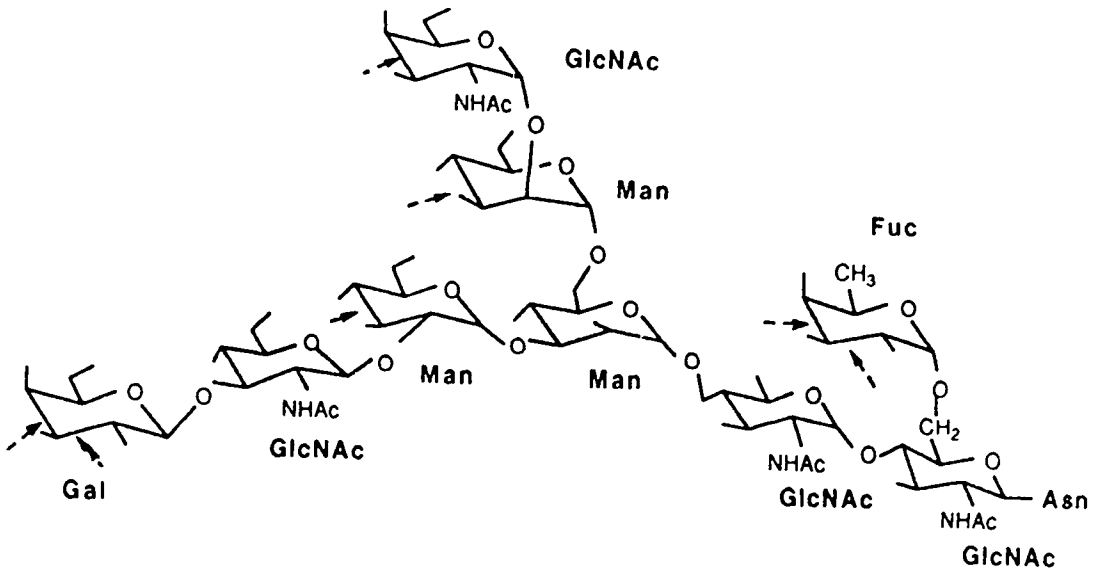


Fig. 4.13 Conformation structure of one (chain I) of the oligosaccharide chains of hIgG molecule. Arrows indicate possible oxidation sites.

4.8.3 DNPH Conjugation

DNPH was used to investigate the influence of several factors on the periodate oxidation of hIgG and MAb 155H.7 and subsequent conjugation with amino or hydrazide compounds. Because of its ready availability and ease of quantitation by UV absorbance, DNPH seemed to be an ideal marker for the study of antibody oxidation. A UV spectrum of DNPH in 0.01 M PBS, pH 7.2 was recorded to calculate the cross absorbance of DNPH at 280 nm, which is also the peak absorbance of proteins (Fig. 4.14). A 40.8% (40.2-41.3) of cross absorbance at 280 nm was calculated by dividing A_{280nm} by A_{360nm}. Thus it was deemed possible to measure the conjugation efficiency of DNPH to oxidized carbohydrate residues on antibodies and proteins after appropriate cross absorbance correction.

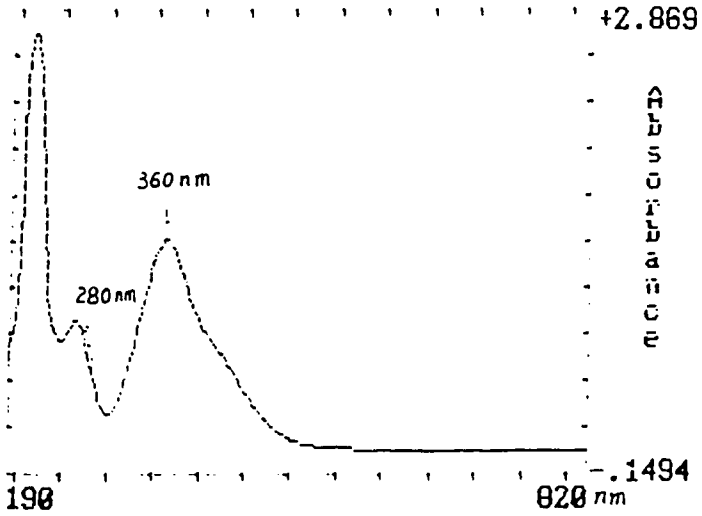


Fig 4.14 UV spectrum of DNPH

4.8.4 Effect of Some Factors on Conjugation of DNPH to antibodies

Two antibodies, hIgG and MAb 155H.7 were used as model compounds in this study. It can be seen from Table 4.13 and Fig. 4.15 that increasing the amount of DNPH in the reaction mixture also increases the incorporation of DNPH. A precipitation occurred when a large amount of DNPH (e.g. >250:1) in DMSO was added.

Table 4.13 Effect of starting ratio of DNPH to hIgG on conjugation

Starting[a] Ratio (DNPH/hIgG)	DNPH residues per hIgG
10	1.41 (1.3-1.5)[b]
60	4.96 (4.1-5.9)
150	9.01 (8.9-9.1)
200	9.59 (9.2-10.0)

a: Oxidation was performed in 30 mM of periodate, 28°C for 30 minutes. Conjugation was carried out at 4°C for 15 hours

b: Average of two samples. Range expressed in parentheses

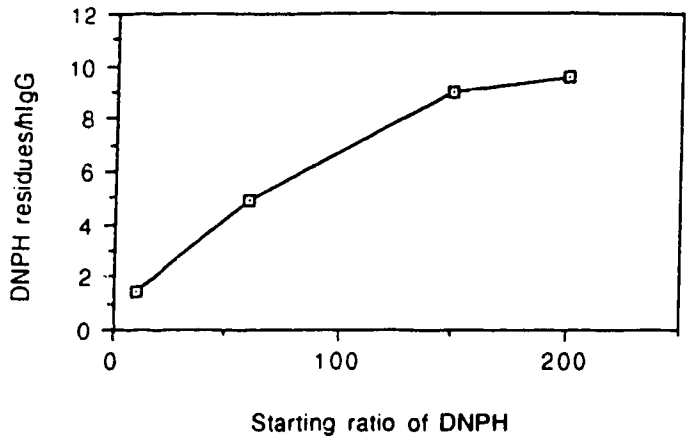


Fig. 4.15 Effect of the starting ratio of DNPH/hIgG on the DNPH conjugation

Time course of the conjugation reaction is given in Table 4.14 and Fig. 4.16. Twelve to 15 hours were needed to reach equilibrium, but shorter reaction times could be used if a smaller amount of incorporation is desired.

Table 4.14 Effect of periodate concentration and reaction time on conjugation of DNPH to antibodies

Conjugation	DNPH/hIgG[a,b]		DNPH/MAb155H.7[a,b]	
Time (hr)	10mM	30mM	10mM	30mM
1	3.4 (3.3-3.5)	4.2 (4.1-4.5)	4.5 (4.0-5.0)	5.0 (4.7-5.3)
3	6.8 (6.6-7.0)	7.2 (7.1-7.3)	6.5 (6.1-6.9)	7.8 (7.5-8.1)
6	8.2 (8.2-8.4)	8.8 (8.7-8.9)	8.5 (8.3-8.7)	9.3 (9.1-9.5)
15	9.3 (9.1-9.5)	10.3 (9.7-10.9)	8.8 (8.6-9.0)	9.5 (9.2-9.8)

a: Average of two replicates, range in parentheses

b: Concentration of $NaIO_4$

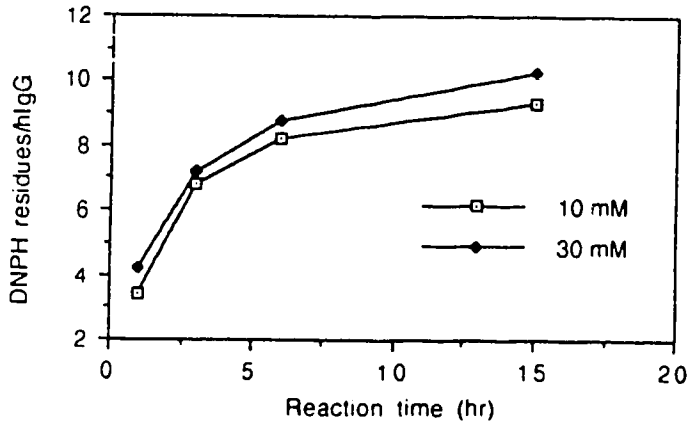


Fig. 4.16 Effect of reaction time on DNPH conjugation reaction

Effect of the concentration of $NaIO_4$ on the incorporation of DNPH is presented in Table 4.15 and Fig. 4.17. It can be seen that higher concentration of oxidant resulted in a higher incorporation of DNPH, indicating a higher degree of periodate oxidation of the antibody. There was no incorporation of DNPH observed when native hIgG was used as a control (Table 4.15). Periodate oxidation of HSA, which served as a non-glycoprotein, was investigated for the possible oxidation of some of the amino acids on the protein. Under the conditions of equilibrium (e.g. 200 fold excess reagent and 15 hours reaction time), 0.15 and 0.2 DNPH residues were incorporated per HSA molecule at 4°C in 10 and 30 mM of $NaIO_4$ respectively. These results suggest that periodate oxidation under controlled conditions could be directed mainly to the carbohydrate moieties of glycoproteins.

Table 4.15 Effect of oxidant concentration on the conjugation of DNPH to hIgG

Concentration of $NaIO_4$ (mM)	DNPH/hIgG Starting ratio	Residues/hIgG[a]
0.1	200	0.9 (0.8-1.0)[b]
1	200	3.2 (3.0-3.4)
10	200	8.1 (7.8-8.4)
30	200	9.8 (9.4-10.2)
0	200	0

a: 15 hours of conjugation reaction,

b: An average value of two data, range in parentheses

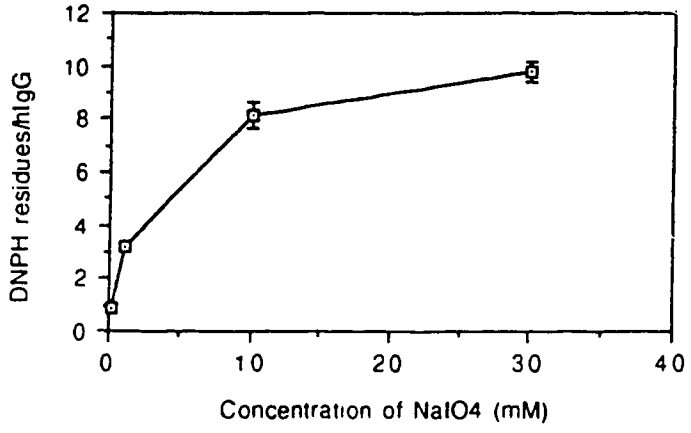


Fig. 4.17 Effect of concentration of $NaIO_4$ on the conjugation of DNPH to hIgG

Table 4.16 gives the results of the correlation of the number of non-reducing sugars on proteins as measured by the number of DNPH from our experiments. It is observed that there are 8-9 DNPH residues incorporated onto hIgG and MAb 155H.7 molecules and that this number is approximately equal to the number of oxidizable sugar residues theoretically predicted for an IgG molecule (section 4.8.2). Ghose, et al. (1988) observed in their experiment during conjugation of an antifolate, methotrexate (MTX), to antibodies that 6-9 moles of INH (isonicotinic acid hydrazide) and 10-12 MTX residues were incorporated per mole of IgG, although about 20 oxidative sites were formed. Barry and Mitchell in a series of papers described the condensation of some hydrazide compounds with periodate-oxidized starch (Barry and Mitchell, 1953a,b; 1954a,b). Their findings showed that only one molecule of reagent had condensed with each potential dialdehyde group. A cyclic structure proposed for these condensation products (Fig. 4.18) supports their observation. A further discussion of this reaction scheme is given in section 4.11.3 of this manuscript.

Table 4.16 Correlation of the number of non-reducing terminal sugars on proteins as measured by the DNPH conjugation with those of theoretical calculation and phenol-sulfuric assay [a]

Proteins	DNPH residues	Theoretical number
hIgG	9.5 (9.0-10.0)	7+6[b]
MAb 155H.7	8.9 (8.6-9.1)	7+6[b]
		Phenol-sulfuric acid Method
HSA-Tα[c]	19.9 (19.4-20.4)	21

a: 30 mM of periodate, 28°C for 30 minutes. 15 hours of conjugation

b: Seven terminal sugars and six innerchain sugars

c: A neoglycoprotein with conjugated disaccharides, Gal-GalNAc (Gal terminus)

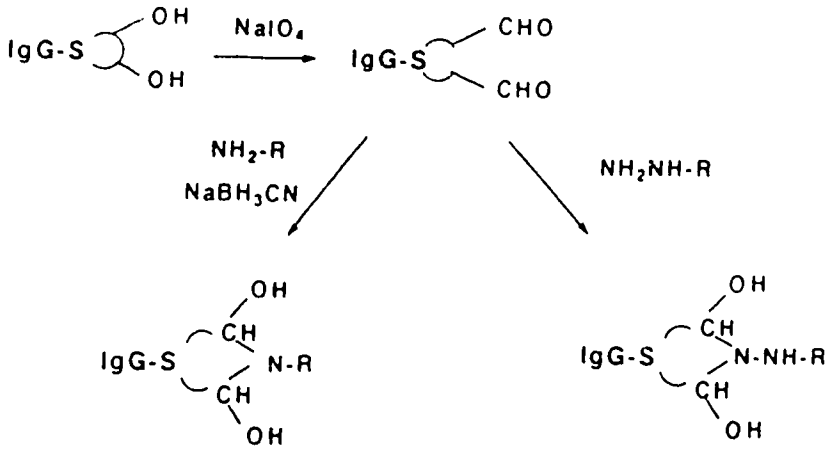


Fig. 4.18 Proposed cyclic structure of the condensation reaction after periodate oxidation of carbohydrate residues on hIgG

4.8.5 Linearity

The assumption that a straight line would be drawn when plotting number of DNPH/glycoprotein vs the S value has been presented in section 3.5.6.1. A calculated slope of the line for HSA-Tα, according to the equation (3.9) (section 3.5.6.1), was around 3.73. In this experiment, all measurements were performed in duplicates using the same batch of the oxidized HSA-Tα. A straight line with r of 0.99 and a slope of 3.78 was obtained from experimental data (Fig. 4.19), suggesting a good correlation between S value and the number of DNPH.

HSA-Tα is a disaccharide, Galß1,3GalNAc conjugated protein, with only terminal Gal available for periodate treatment. From the previous discussion, if one Gal reacts with one DNPH, the number of DNPH residues per HSA-Tα molecule, therefore, will represent the number of disaccharides coupled onto the HSA molecule. It was found that there were 19-20 DNPH residues, corresponding to the amount of disaccharide, Tα, on HSA-Tα molecule. This was in good agreement with the number of 21-22 residues of Tα per HSA molecule as assayed by phenol-sulfuric

acid method (Table 16). In the case of antibodies, such as hIgG and MAb 155H.7, there are about 6-7 terminal sugars and 5-6 innerchain sugars which could possibly be oxidized depending on the reaction conditions. Eight to nine DNPH residues were conjugated per antibody molecule under the conditions used in this study, thus providing a consistent correlation with the number theoretically predicted. Because of the complexity of the carbohydrate composition and configuration on natural glycoproteins, the actual relation between amount of reagent (DNPH here) and number of sugars which are vulnerable to periodate oxidation is the subject of speculation. However, the method may be particularly useful in measuring the sugar content of simple sugar conjugates in areas such as neoglycoprotein research. One must be cautious of the inherent limitations when applying the same principles to complex oligosaccharide chains of unknown composition which are attached to macromolecular structures.

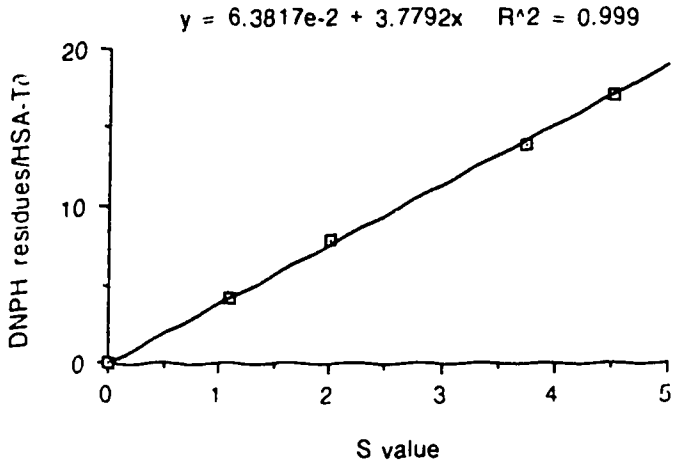


Fig. 4.19 Plot of the number of DNPH/HSA-Tα *vs* the S value

4.9 Purification and Chelation Study of a Novel Chelating Agent, PNB-PDTA Synthesized According to Fig. 3.4

The problem encountered in the purification of the chelate which was synthesized according to the scheme in Fig. 3.4 was that several side products existed in the crude product in which the amount of desired product was quite small. Efforts were made to purify it by AG 1-X8 anion exchange, Bio-Gel P-2 and preparative TLC chromatography and were not successful in that TLC and the NMR data continued to reveal impurities. Therefore preliminary chelation studies were carried out on the purified fractions to yield some indication as to whether or not pursue further experiments on PNB-PDTA.

4.9.1 Chelation with ^{111}In

^{111}In chelation of PNB-PDTA was obtained in an average of 97.2% radiochemical yield after 15 minute reaction at room temperature as assayed by TLC with 10% NH_4OAc/MeOH 1/1 (v/v) solvent system. Table 4.17 gives the results obtained after PNB-PDTA in 0.1 M HCl aqueous solution was mixed with fresh $^{111}InCl_3$.

Table 4.17 Chelation study of PNB-PDTA with $^{111}InCl_3$

Amount of ^{111}In	Amount of PNB-PDTA	% Yield[a]
30 μCi	20 μg	96.2 (2.3)
30 μCi	30 μg	97.1 (1.6)
30 μCi	100 μg	98.5 (1.3)

a: n=2, S.D. in parentheses

The above results indicated to us that the novel chelating agent, PNB-PDTA was a useful chelator for ^{111}In and further investigation into optimization of the purification, the labeling conditions, and conjugation of the chelate to antibodies were warranted.

4.9.2 Chelation with ^{99m}Tc

Preliminary experiments were also conducted to explore the possibility of using FSA as a reductant for the labeling of PNB-PDTA with ^{99m}Tc. Since there was concern that there may be competition for the metal ions to bind to the limited chelation sites on the protein conjugates, FSA was used as an alternative. FSA is a reducing agent used in the preparation of some of ^{99m}Tc-labeled radiopharmaceuticals such as ^{99m}Tc-glucoheptonate (Jette, et al., 1982). The concentrated FSA solution is slightly turbid (Fritzberg, et al., 1977). Thus the FSA solution used in our study was prepared by adding 10 mg of FSA to 1 mL of DD-H_2O and shaken vigorously. The solution was then filtered with Millipore filter (0.22 μ) after 20 minutes and used for the reduction study of ^{99m}Tc. Since the exact amount of dissolved FSA was not known, relative volumes of the solute were used in the following studies.

Reactions were performed by mixing 400 μL of FSA solution and 40 μg of chelate at pH 8.5. Whatman No.1 paper chromatography developed in 0.1 M NaOAc/CH_3CN 10:1 (v/v), at pH 6.0 was used for the analysis of the labeled products. Sodium pertechnetate had a R_f value of 0.75 while chelated ^{99m}Tc moved to the solvent front. Colloids or other impurities stayed at the origin. At room temperature, the maximum observable labeling was 50-60 %. Higher reaction temperatures were needed for complete chelation. Table 4.18 and Fig. 4.20 show the effect of temperature on the chelation reaction. When the reaction was conducted at 65°C, almost quantitative labeling yields (95 %) were obtained after 10 minutes. On the other hand, it took ten times longer to achieve the same labeling yield when the reaction was conducted at 37°C. Fritzberg (1977) reported that

heating was necessary when FSA was used as a reductant. Their results showed that a 99% labeling yield of the ^{99m}Tc-DTPA prepared with FSA was obtained at pH 7.5, 58°C after 20 minutes.

Table 4.18 Effect of temperature on the chelation reaction of ^{99m}Tc

Temperature (°C)	pH	FSA (μL)	% Yield[a]
25[b]	8.5	400	55.0 (8.9)
37[b]	8.5	400	95.0 (1.2)
65[b]	8.5	400	95.3 (1.1)

a: n=3, S.D. in parentheses

b: Reaction time was 120 minutes

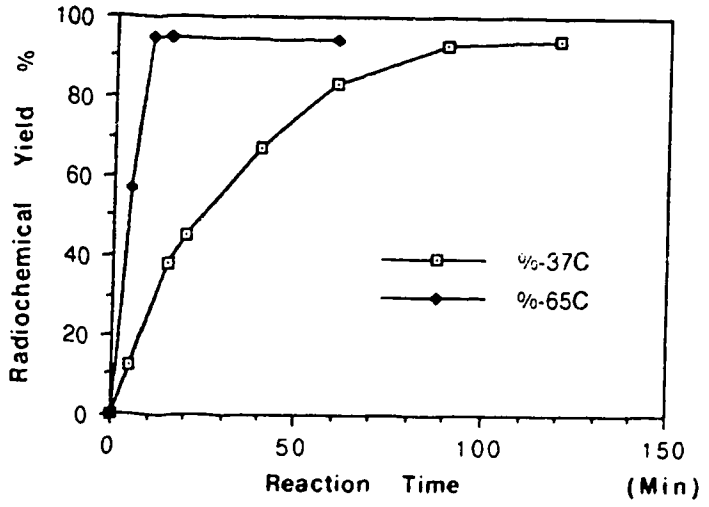


Fig. 4.20 Effect of reaction temperature on ^{99m}Tc-labeling time course. Each point represents an average of two runs.

The influence of solution pH on the labeling with ^{99m}Tc was carried out using 400 μL of FSA solution, 40 μg of chelate at 37°C for 1 hour. As shown in Fig. 4.21, basic pH had a favourable influence on the labeling yield. These results were in agreement with the findings of Fritzberg, et al. (1977) and Jette, et al. (1982). A proposed reduction mechanism of FSA is presented in Jette's paper which can be summarized as follows: (1) FSA is a strong reducing agent in an alkaline medium; the reduction potential is dependent on hydroxide concentration and temperature. (2) the products of the dissociation of FSA under catalysis of hydroxide are urea and bisulfite (HSO_3^-).

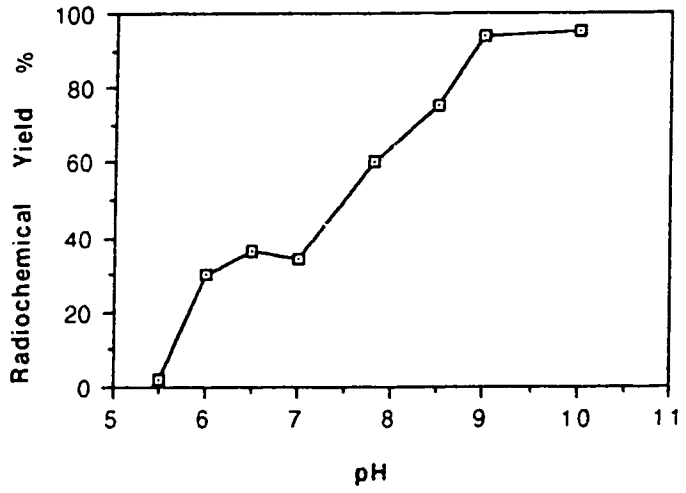


Fig. 4.21 Effect of solution pH on ^{99m}Tc labeling. Each point represents the results of two runs

The effect of the amount of FSA on the labeling yield is given in Fig. 4.22. A certain amount of FSA (about 300 μL) is needed to reach a high labeling yield, while lower FSA quantities resulted in incomplete labeling. In general, the efficient conditions necessary for the labeling of PNB-PDTA are: a high reaction temperature of 65°C, a volume of FSA larger than 200-300 μL, and a pH greater than 9. These conditions could be adopted for use with different chelating agents

with slight modification. Because of the limitations imposed by these conditions, particularly the high temperature (65°C) and high pH (>9), it was felt that their application towards the labeling of sensitive proteins such as antibodies would result in unacceptable denaturation of the biologicals. Consequently alternative methods were explored to achieve the desired labeling of monoclonal antibodies.

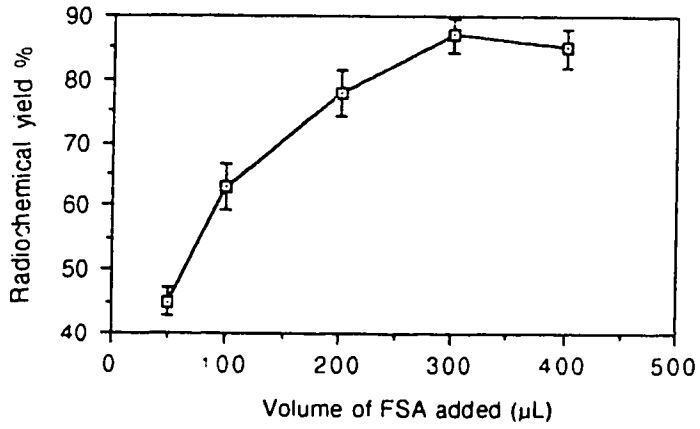


Fig. 4.22 Effect of the amount of FSA on ^{99m}Tc labeling

4.10 Preparation and Chelation Study of a Novel Chelating Agent, p-NH_2-Bz-PDTA (PAB-PDTA)

The characterization data of PNB-PDTA (p-NO_2-Bz-PDTA), the precursor compound is as follows: (Dr. V. Guay's data)

IR:νmax. (KBr) 2940br (CO_2H), 2623br (N^+H), 1743 (CO_2H), 1663 (CO_2H), 1552 (NO_2), 1350 (NO_2), and 1226 cm^{-1} (CO_2H).

NMR: δ (D_2O)+DCl;TSP) 2.91 (1H, m, CH), 3.22 (2H, d, J 7.53, $ArCH_2$), 3.58 (2H, dd, J 14.03 and 5.82 Hz, 2 x CHC*H*HN), 3.92 (2H, dd, J 14.03) and 5.43 Hz, 2 x CHCH*H*N), 4.38 (2H, d, J 17.41 Hz, 2 x NC*H*HCO), 4.48 (2H, d, J17.41 Hz, 2 x NCH*H*CO), 7.61 (2H, d, J 8.69 Hz, 2 x ArH), and 8.27 (2H, d, J 8.69 Hz, 2 x ArH).

An e.i.-m.s. Found: C, 48.69; H, 5.20; N, 9.37. $C_{18}H_{23}N_3O_{10}$ requires C, 48.98; H, 5.25; N, 9.52%.

PAB-PDTA obtained by hydrogenation of PNB-PDTA was characterized by ^{1}H-NMR spectrum (300 MHz, D_2O) (Appendix 12) with signals δ_1 7.0 (d, 2H) and δ_2 6.7 (d, 2H). Both ninhydrin color and UV absorbance were used to detect and confirm the presence of both primary amino group and aromatic structure. Ninhydrin colour was positive for amine derivative and negative for nitro derivative. Silica gel TLC of PAB-PDTA had one spot at R_f 0.2 in EtOH/CH_3CN 4/1 (v/v); R_f 0.6 in CH_3CN/H_2O 7/3 (v/v); R_f 0.24 in EtOH/NH_4OH 4/1 (v/v) systems. Because only one spot was observed by TLC in the above 3 solvent systems, therefore, a purity of PAB-PDTA of 90 % or greater was assumed.

Time course of ^{111}In-labeling yield was also investigated (Fig. 4.23). The results obtained from the experiment indicated that PAB-PDTA was a good chelator for ^{111}In and that chelation was essentially complete within 5 minutes.

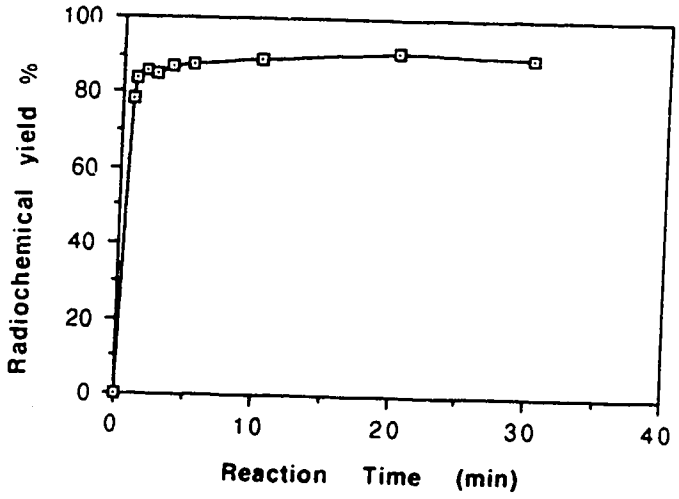


Fig. 4.23 Time course of ^{111}In-labeling of PAB-PDTA

When the amount of ^{111}In activity was increased in relation to the quantity of PAB-PDTA, it was noted that the resultant radio-chemical yield was slightly decreased (Fig. 4.24). This could be due to the presence of contamination of trace

metal ions in the ^{111}In solution, thus highlighting the importance of using fresh preparations of $^{111}InCl_3$ for this type of experiments. This was indeed quite evident by the failure of weeks-old preparations of ^{111}In-Cl_3 in labeling experiments, possibly due to continuous leaching of trace metal ions from the glass container in the solution and decay product of ^{111}In.

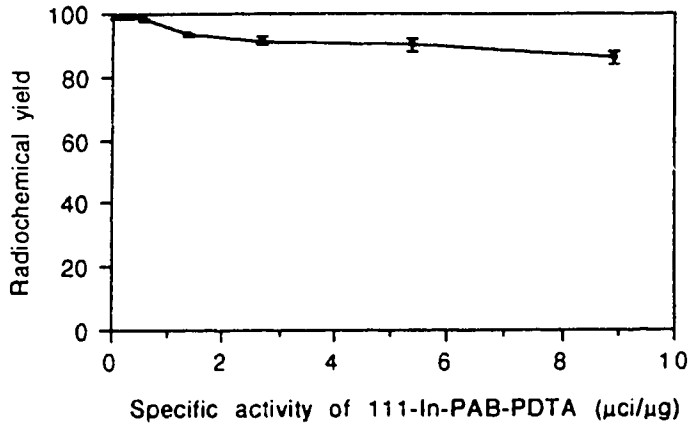


Fig. 4.24 Study of specific activity of ^{111}In-labeled PAB-PDTA

4.11 Conjugation of PAB-PDTA to the Carbohydrate Moiety of Antibodies

4.11.1 Conjugation and Reduction of Schiff Base

Periodate oxidation of antibodies has been investigated and discussed in previous sections. Oxidized antibodies were reconstituted to a concentration of 2-3 mg/mL. A pre-determined amount of PAB-PDTA in excess of 50-700 fold was added to the protein solution and followed by $NaCNBH_3$. The reaction mixture was separated by gel filtration chromatography. Subsequent addition of ^{111}In-citrate resulted in a 95% protein bound radioactivity, as assayed by a Sephadex G-50 column, SE HPLC, and 99% radioactivity stayed at the origin as assayed by ITLC at a starting ratio of PAB-PDTA to oxidized hIgG of 50:1. Fig. 4.25 shows the

results of ^{111}In-labeled PDTA-hIgG conjugate (at 50:1 ratio, 4°C for 12 hours) and ^{111}In-labeled native hIgG as evaluated by HPLC and monitored for UV absorbance and radioactivity. The elution profile of ^{111}In-labeled conjugate clearly indicated that radioactivity was associated with the antibody conjugate. The secondary peak from the UV absorbance traces was associated with the citrate buffer. There was no radioactivity associated with the control native antibody, indicating the absence of non-specific binding of the ^{111}In to the native antibody.

An experiment was conducted using the same batch of oxidized hIgG (30 mM of [illegible] 8°C for 30 min) in duplicate to investigate the reaction time course. Ti[illegible]io n of the number of residues per antibody molecule was carried out i[illegible] of a known amount of PNB-PDTA as assayed by ITLC. Results, summarized in Table 4.19, indicate that chelate incorporation could be achieved at short time intervals although higher incorporation would require a longer reaction time. As much as 5 chelates residues could easily be conjugated after a 15 hour reaction at 4°C.

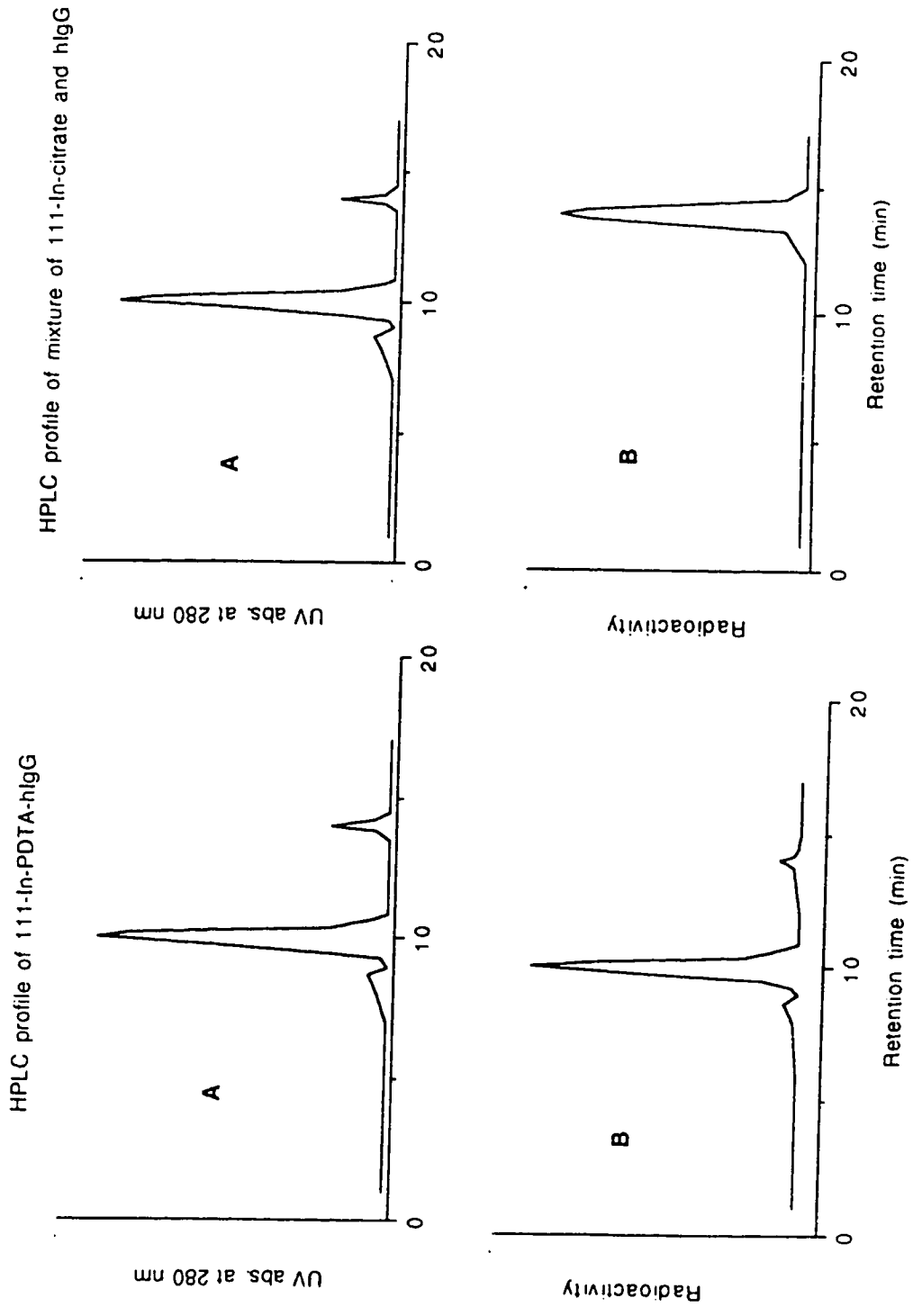


Fig. 4.25 HPLC elution profile of ^{111}In-labeled PDTA-antibody conjugate and native antibody control

Table 4.19 Time course of PAB-PDTA conjugation with oxidized hIgG

Conjugation Time (h)	O-hIgG[a] (μg/μL)	Chelate/Ab Ratio	$NaBH_3CN$ (mM)	Residues[b] Per Ab
0.5	2.1	230	10	0.9 (0.1)
2	2.1	230	10	2.8 (0.8)
4	2.1	230	10	3.9 (1.2)
15	2.1	230	10	4.5 (0.7)

a: Oxidized hIgG (30 mM of $NaIO_4$, 4°C for 30 minutes)

b: Results from two runs assayed by ITLC. S.D. in parentheses

4.11.2 Exploration of Non-specific Binding of Oxidized hIgG by ^{111}In Citrate

The percentage of protein-bound ^{111}In was measured using a Sephadex G-50 gel filtration column for selective preparations. Routine analysis was usually done by ITLC in a solvent system consisting of acetone/saline 16/1 (v/v), pH 2.0 adjusted by 0.1 M HCl. ^{111}In-citrate and the mixture of ^{111}In-citrate with native antibody gave an R_f value of 0.6-0.7, ^{111}In-PDTA had an R_f of 0.8-0.85, while ^{111}In-labeled PDTA-antibody conjugate was retained at the origin in this system.

The effect of antibody periodate oxidation and possible reactivity with ^{111}In-citrate preparation was explored. In this case, the oxidized hIgG (10 mM $NaIO_4$, 30 minutes) was first purified by a G-50 column to remove excess $NaIO_4$ and the eluate was then lyophilized. The oxidized hIgG, reconstituted with DD-H_2O, was mixed with a solution of ^{111}In-citrate to about 0.5 μCi/μg. After a period of 15 minutes the mixture was then subjected to analysis using ITLC and G-50 chromatography. In the former case, it was found that the radioactivity was quantitatively associated with the

antibody at the spot of origin. These surprising results were compared, under the same chromatography conditions, to a preparation of unmodified hIgG which was mixed with ^{111}In-citrate and the radioactivity was found to quantitatively migrate to an Rf of 0.7. This seems indicate that the oxidation of hIgG resulted in some form of direct binding to the ^{111}In-citrate. The TLC results are summaried in Table 4.20.

Table 4.20 Analysis results of TLC chromatography

Chelates	ITLC or Cellulose (Rf)[a]
^{111}In-citrate	0.65
^{111}In-PAB-PDTA	0.75
^{111}In-citrate + Ab[b]	0.65
^{111}In-citrate + O-Ab[c]	0.0-0.1
^{111}In-PDTA-Ab	0.0-0.1

a: Acetone/saline 16/1 for ITLC and MeOH for cellulose, pH 2 adjusted using 0.1 M HCl for both

b: Native hIgG and native MAb 155H.7

c: Oxidized hIgG and MAb 155H.7

The nature of this binding and its stability was further explored by transchelation experiments using 50-fold excess PAB-PDTA or EDTA. The results observed after ITLC or cellulose TLC separation are given in Table 4.21. After 24 hours, 78% radioactivity was associated with the spot at the origin in the case of PAB-PDTA, while in the case of EDTA displacement, 98% of the radioactivity was still associated with the spot at the origin after 48 hours. Attempts to find a better separation system with ITLC and cellulose TLC were not successful.

Table 4.21 Transchelation test of non-specific binding of ^{111}In-citrate to oxidized hIgG[a]

Chelates	Displacement time(hr)			
	0	12	24	48
	% Protein bound[c]			
PAB-PDTA (50:1)[b]	98	95	78	70
EDTA (50:1)[b]	98	98	98	98
Native hIgG[d]	0	0	0	0

a: 10 mM of $NaIO_4$, at 28°C for 30 minutes

b: Chelate to oxidized hIgG ratio

c: Assayed by ITLC or cellulose TLC

d: Mixture of native hIgG with ^{111}In-citrate

However, when Sephadex G-50 chromatography separation was applied in the absence of chelate challenge, it was noted that almost all of the radioactivity was removed from the antibody molecule. This finding is of importance when different analytical techniques are applied to measure radioactivity bound to modified antibodies. Therefore, the ^{111}In may have been non-specifically associated with the protein and readily removed by simple dilution during gel chromatography.

4.11.3 Estimation of Number of Chelates Incorporated per Antibody Molecule

For the determination of the number of chelate residues, purified hIgG-PDTA conjugate was mixed with a known amount of PNB-PDTA and then a fixed

amount of ^{111}In-citrate was added to the mixture. Analysis of the percentage of the protein-bound radioactivity which was needed in the calculation of the number of chelate residues (equation 3.10 in section 3.9.3.2) was conducted by Sephadex G-50 chromatography for selected samples. The results of the estimated number of chelate residues for two batches of hIgG-PDTA conjugates are given in Table 4.22 along with the results from ITLC and cellulose analysis for the comparison.

ITLC and cellulose chromatography were also used to determine the protein-bound radioactivity because of the simplicity of the above methods. As discussed in the previous section, the amount of ^{111}In non-specifically bound to oxidized antibodies which had been conjugated to PAB-PDTA contributes to the percentage of the protein bound (P%) since this direct binding of ^{111}In with oxidized antibody stayed at the spot of the origin on ITLC and cellulose strips as well. Two experiments were designed in order to address this issue. In the first experiment, ITLC was used to measure the protein-bound radioactivity for PDTA-conjugates. In the second experiment, an oxidized antibody was mixed with the same amount of PNB-PDTA and the same amount of ^{111}In-citrate was added as that in the first experiment. After ITLC analysis, the amount of radioactivity bound to the antibody was then considered as non-specifically bound and consequently subtracted from the fraction of observed radioactivity associated with the antibody in the first experiment. Despite many obvious drawbacks to this technique, it probably represents a worst-case scenario in our estimate of number of chelates bound to the antibody. The results from this experiment were compared to those from the Sephadex G-50 analysis which, as stated previously, resulted in the removal of all non-specifically bound radioactivity. Table 4.22 shows that indeed comparable results could be obtained from both techniques.

Table 4.22 Estimation of the number of chelate residues

PDTA-conjugate	Sephadex G50	ITLC	Cellulose
Preparation I[a]	4.4[c]	4.2 (4.0-4.4)[d]	3.9 (3.7-4.0)
Preparation II[b]	0.9[c]	1.1 (0.9-1.3)	1.3 (1.0-1.5)

a: Antibody was oxidized in 10 mM of $NaIO_4$, at 28°C for 30 minutes. Chelate to antibody ratio of 50:1 and 10 mM of $NaBH_3CN$ were used and conjugation reaction was carried out at 4°C for 15 hours.

b: One step conjugation at a chelate to hIgG ratio of 20:1, 10 mM of $NaIO_4$, at 4°C for 30 minutes. (see section 4.11.5.2)

c: n=1

d: An average of two data with range in parentheses

4.11.4 Elimination of the Sodium Cyanoborohydride Reduction Step

The conjugation was also investigated in the context of eliminating the final reduction step of the Schiff's base. Oxidation was carried out at 25°C for 30 minutes in 30 mM of $NaIO_4$. Conjugation was conducted in citrate buffer, pH 5.2 at different initial amounts of chelate for 15 hours at 4°C. The experimental results presented in Table 4.23 indicate that incorporation of the chelate was achieved without any subsequent reduction with sodium cyanoborohydride.

Table 4.23 Incorporation of the chelate in a non-reductive conjugation reaction

O-hIgG[a] Concentration (μg/μL)	Starting ratio chelate/hIgG	Chelate/hIgG[b]
5	10	0.6 (0.5-0.8)
5	20	1.0 (0.9-1.1)
5	30	1.6 (1.4-1.7)
5	40	2.0 (1.8-2.1)

a: Oxidized hIgG

b: Analyzed by ITLC and corrected by subtracting the amount of non-specific binding. An average of two data with range in parentheses

As the modification occurs on the saccharide moiety of IgG molecule, the following scheme (Fig. 4.26) is postulated as a possible path for the reactions leading to the relatively stable product.

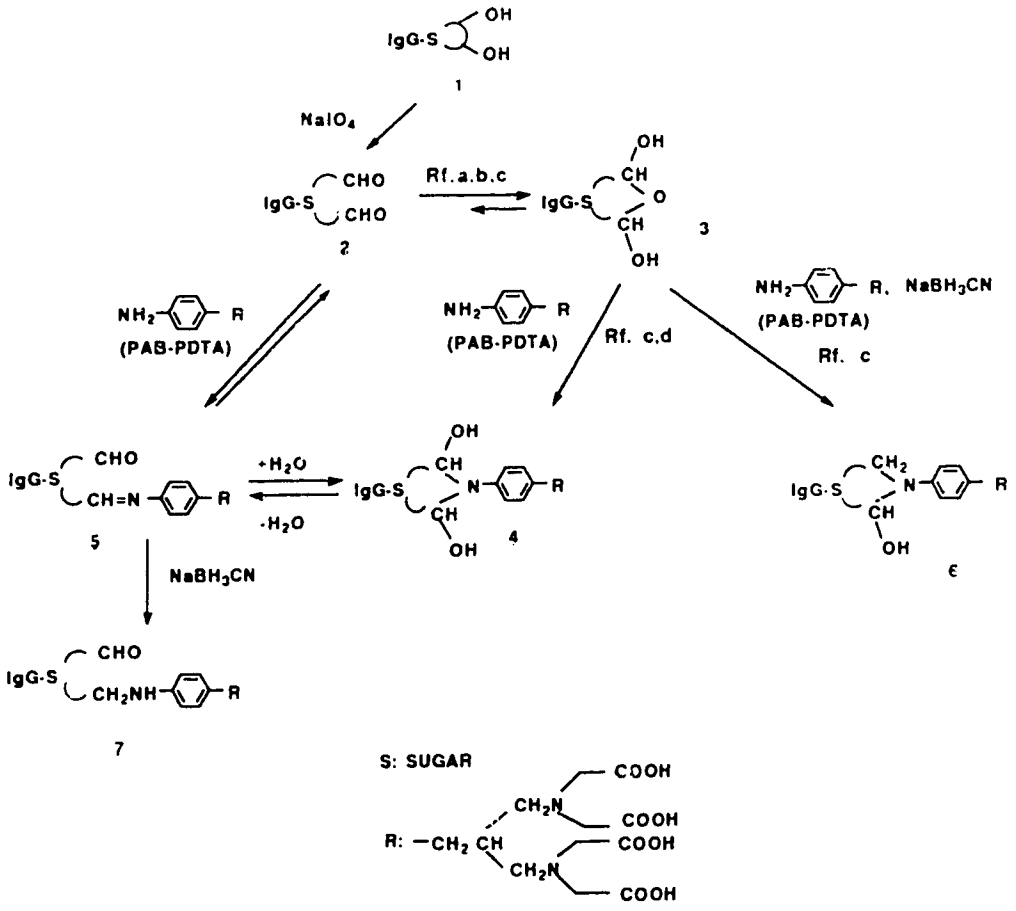


Fig. 4.26 Postulated mechanism of reaction between periodate-oxidized carbohydrates and PAB-PDTA. Ref. a: Rowen, et al., 1951; b: Herd, et al., 1953; c: Barry, et al., 1953; d: Khym, 1963

Because of the lack of evidence for the presence of the aldehyde groups, as deduced from spectrophotometric data, after periodate oxidation of methyl α-D-glucopyranoside and cellulose (Rowen, et al., 1951; Hurd, et al., 1953), one can speculate that only a small amount of dialdehyde product 2 (Fig. 4.26) may actually be present. The evidence of the existence of an extra water molecule in cyclic structure 3 (Fig. 4.26) was obtained from the analysis of the crystalline dialdehyde from four different methylaldohexapyranosides (Barry, et a., 1953a). On the other hand, stability of the non-reduction product 4 (Fig. 4.26) was found to be dependent on the amine used and the pH of the solution. For example, a study of the reaction of methylamine with periodate-oxidized adenosine 5'-phosphate (Khym, 1963) indicated that the addition complex was stable in the pH range of 3 to 8. Another example was reported by Barry and Mitchell (1953a,b) by studying the reaction of oxidized starch and p-aminobenzoate. The precipitate resulting after acidification with dilute acetic acid contained nearly one aminobenzoic acid residue per glycol group. In the form of 5 (Fig. 4.26), the C=N double bond was stabilized by the resonance effect of the aromatic ring structure, although the majority form of the non-reduction was product 4 (Fig. 4.26). However, because of the instability at lower or higher pH, one could speculate that some form of dissociation of the chelate from the antibody would take place intracellularly where the pH in certain cell organelles is acidic.

The reduction of 3 (Fig. 4.26) results in a more stable product 6 (Fig. 4.26). For example a morpholine derivative, obtained after reduction of addition complex of methylamine with oxidized adenosine 5'-phosphate by $NaBH_4$, did not hydrolyze in 0.25 M HCl (Khym, 1963). While the C=N double bond in Schiff base was reduced by $NaBH_3CN$ to 7 (Fig. 4.26).

Another consideration is that a cleavable linkage between chelates and antibodies may facilitate the clearance of labeled antibody from circulation thereby reducing the background level during radioimmunoscintigraphy. On the other hand, one of the disadvantages of the use of $NaCNBH_3$ as a reducing agent is the toxicity

associated with the cyanate compound. Elimination of excess $NaCNBH_3$ from the reaction solution becomes imperative if the compound is to be used *in vivo*. This adds an additional step in the manipulation of the antibody which could result in loss of immunoreactivity.

4.11.5 One Step Conjugation of PAB-PDTA to Antibodies

One stated advantage of the utilization of the carbohydrate moiety of antibodies as a modification site is the preservation of the antibody immunoactivity since the site of carbohydrate attachment, the CH2 of the Fc portion, is not involved in antibody-antigen binding activity. However, the conjugation strategy currently used in industry is a multiple step approach involving two steps of gel filtration separation or dialysis and a longer handling time.

Our investigation of conjugation of PAB-PDTA to the oxidized carbohydrate moiety of antibodies has demonstrated the possiblity of elimination of $NaBH_3CN$ reduction step and a reduction in the conjugation time. Based on these results, we explored the idea of a one-step oxidation/conjugation as a method of producing a useful antibody-chelate conjugate.

4.11.5.1 Study of Periodate Oxidation of PAB-PDTA

Because of the risk of possible oxidation of the chelating agent, PAB-PDTA itself, an investigation was carried out to minimize unwanted oxidation. It was found that when periodate oxidation of PAB-PDTA was performed at 28°C with quantities larger than 30 mM of $NaIO_4$, a red to brownish colour solution was obtained. TLC results showed several spots and a long tail from the spot of origin to R_f 0.5, indicating the presence of several oxidation products. When the oxidation was carried out in 10 mM of $NaIO_4$, at 4°C in the dark, only slight reddish color was observed. TLC results showed one major spot, R_f 0.24 in $EtOH/NH_4OH$ 4/1 (v/v) system and R_f 0.6 in CH_3CN/H_2O 7/3 (v/v), these were the same as those observed for the starting material, PAB-PDTA. Both observation under UV light as well as a positive ninhydrin reaction on the plate indicated the presence of an intact chelate

molecule with both a primary amine group and an aromatic ring strucure. ^{1}H-NMR spectum of PAB-PDTA (Fig. 4.27) also illustrated the integrity of the aromatic protons of the chelate, δ 6.7 (d, 2H) and δ 7.0 (d, 2H), except for the presence of two small peaks observed at δ 6.9 and δ 6.95, indicating a small amount of oxidation products of PAB-PDTA after exposing the chelate to periodate under mild oxidation conditions.

One of the oxidation products from peroxidate oxidation of aromatic amine is a nitroso compound (Plesnicar, 1978). We assumed that a similar reaction occurred during periodate oxidation. Thus, condensation among a nitroso derivative and the aniline derivative may be partly responsible for the colour formation even under the mild oxidation conditions used in this study. Other oxidation products may be the result of the oxidation of the benzyl carbon in PAB-PDTA structure as well. Tertiary nitrogen is unlikely to be oxidized by periodate (Nicolet, 1939). Although the imine formed may be oxidized to oxaziridine by peroxy acid (Plesnicar, 1978), the reaction is unlikely to occur under the mild periodate oxidation applied in the present investigation. It was also reported that the nitroso aromatics were unlikely to react with an aldehyde (Corbett, 1981).

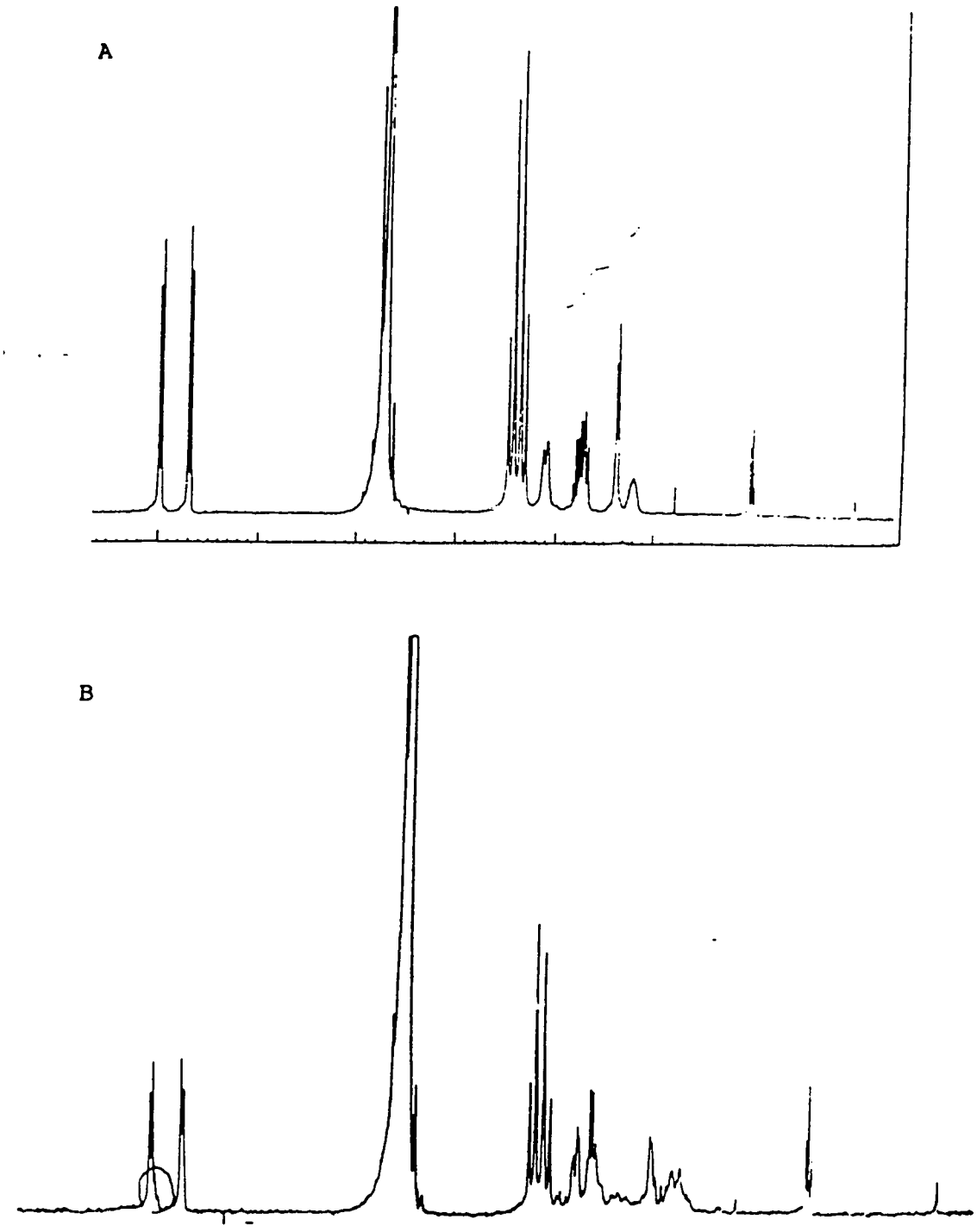


Fig. 4.27 ^{1}H-NMR spectra of PAB-PDTA (A) and the PAB-PDTA exposed to periodate under mild conditions (B)

In summary, the above results implied that only very little periodate oxidation of the chelate occurred under controlled mild conditions, and that the subsequent proceeding of one step conjugation was insured, even though the course of other related reactions have not been investigated.

4.11.5.2 One Step Conjugation and ^{111}In-labeling

One step conjugation of PAB-PDTA to hIgG and MAb 155H.7 was performed in 10 mM of $NaIO_4$, at 4°C for 30 minutes at a starting ratio of 20:1 in NaOAc buffer, pH 5.2. The resultant light red color in the reaction mixture was immediately separated by a gel filtration column after completion of the reaction, and the eluant collected from the column was freeze-dried and labeled with ^{111}In-citrate. The number of chelate residues incorporated on hIgG, as determined by Sephadex G-50 chromatography and TLC chromatography, is given in Table 4.22 in preparation II. About one PDTA was incorporated on hIgG in a 30-minute reaction. A greater than 90% protein bound radioactivity was observed in one experiment, as assayed by size exclusion HPLC (TSK 3000SWXL) and Sephadex G-50 chromatography.

An investigation of one step conjugation of DNPH to antibody was also performed as the determination of the number of DNPH residues was much easier. Although investigation of the possible oxidation of DNPH itself was not performed, a postulation was made based on the similarity in structure between the amine and hydrazine compound. The -NH- group was even less reactive with periodate than NH_2- group, since the tertiary nitrogen was not oxidized by periodate (Nicolet, 1939). An average of 3.6 (S.D. 0.3) DNPH residues was conjugated per hIgG molecule in the reaction of 10 mM of $NaIO_4$, at 4°C for 30 minutes at a starting ratio of 100:1 of DNPH to hIgG. From the results of our previous investigation, the condensation reaction of DNPH (a hydrazine) with oxidized hIgG was the rate limiting step since the oxidation reaction proceeded much faster. One-step conjugation, because of its simplicity, is particularly useful when a lower incorporation of chelate residues is desired. Under certain conditions in

radioimmunoscintigraphy, an average of one chelate residue per antibody molecule is enough for the radiolabeling of the molecule when specific activity (μCi/μg of conjugate) of the labeled antibody is not of great concern whereas preservation of the immunoreactivity of antibodies is of more importance.

4.11.6 Assessment of Formation of Oligomers of the Chelate-Antibody Conjugate and the Transchelation properties of ^{111}In-labeled Conjugates

It is of importance to monitor the formation of oligomers and dimers during chemical manipulation of antibodies because of the potential of their accumulation in the liver, spleen and other RES tissues. The presence of these aggregates was assessed by Sepharose CL-4B chromatography or HPLC. There were no additional oligomers detected arising from the manipulation (reduction by $NaCNBH_3$, no reduction and one step) except for the 5 to 10 % aggregates originally present in the purchased hIgG preparation (data see Table 4.2 and Table 4.3 in section 4.2). Intermolecular aldol condensation was not detected as evidenced by the above analysis results. However, we are unable to evaluate the intramolecular condensation since in the latter case, the small change in molecular weight could not be detected by these analytical methods. Intramolecular condensation resulting from the periodate oxidation process has been reported with the analysis of heavy chains and light chains by electrophoresis (Brown, et al., 1986).

The ability of ^{111}In radioactivity to be retained by the chelate on the protein molecule was studied by means of transchelation to free PDTA and EDTA. Table 4.24 shows the displacement test results of ^{111}In-labeled chelate conjugates prepared by the one step method and after reduction with $NaBH_3CN$ and labeling with ^{111}In-citrate. ^{111}In-labeled conjugates, analyzed by ITLC after being challenged with 50-fold excess PAB-PDTA and EDTA showed no reduction in protein bound radioactivity after 2 days. This data demonstrates that PDTA-hIgG prepared with or without a reduction step and PDTA-MAb 155H.7 prepared without a reduction step are stable to transchelation as determined by ITLC. This information is not relevant to assess the stability of the chelate-protein bond. This could have been

studied by measurement the loss of chelate during incubation in solution at varous pH values or in serum as a function of time. However, *in vivo* stability of the PDTA-conjugates will be discussed in section 4.11.9.

Table 4.24 Transchelation analysis of chelate-antibody conjugates

Conjugates	Chelate added (50:1)	Incubation time (hr) 0	12	24	48
		% Protein bou nd[a]			
One step[b] hIgG	PAB-PDTA	98	98	97	97
	EDTA	98	97	96	97
One step[c] MAb 155H.7	PAB-PDTA	98	97	98	97
	EDTA	98	97	97	97
Reduction[d] hIgG	PAB-PDTA	98	97	97	98
	EDTA	98	97	98	98

a: Analyzed by ITLC

b: About one PDTA residue per hIgG molecule

c: About one PDTA residue per MAb molecule

d: About 4.5 PDTA residues per hIgG molecule

4.11.7 Evaluation of Immunoreactivity of PAB-PDTA Antibody Conjugate by ELISA assay

A human colon carcinoma cell line, LS174T, and HSA were used as sources of antigen for the binding studies of MAb 155H.7. A higher binding of PDTA-antibody conjugate dilutions than that of corresponding dilutions of native MAb 155H.7 to the LS174T homogenate (Table 4.25 and Fig. 4.28) was observed. Since the only modification made to the antibody was that of oxidation followed by conjugation to PAB-PDTA it was possible that the observed increase in binding to LS174T cell homogenate was due to either the increase in carboxylic acid residues on the MAb as a result of conjugation or due to the presence of excess aldehyde or OH- residues as a result of carbohydrate oxidation. HSA was reported to also possess strong binding properties to MAb 155H.7. Since there were no apparent differences in the binding between the PDTA-antibody conjugate and native antibody to HSA, one might conclude that the apparent changes in immunoreactivity after conjugation of PAB-PDTA to MAb 155H.7 shown for the LS174T homogenate were due to an increased non-specific binding in this system. The homogenate is a crude mixture of cellular components and it would not be surprising to find changes in non-specific binding due to interaction with any of the numerous components. In addition, there were no observable differences between the conjugates obtained after the reduction and one-step conjugation processes. The reason for the increase in observable immunoreactivity of the conjugate to the cell homogenate remains to be very speculative. A possible explanation is the increase in hydrophobicity of the antibody due to the introduction of a benzyl group from the PAB-PDTA. Another possibility could be attributed to the effect of periodate oxidation on the conformational structure of the antibody thus resulting in alteration of the binding sites of the variable region. The introduction of additional carboxylic groups from DTPA to this antibody is also known to increase the apparent immunoreactivity (personal communication, Dr. T. R. Sykes). Due to the nature of the assay, it is also possible that the second (enzyme-labeled) antibody shows increased binding to the modified MAb 155H.7 than to the native form, however,

the difference in the LS174T homogenate and albumin study does not support this conclusion. Thus preservation of the immunoreactivity of the MAb treated *via* manipulation of the carbohydrate moiety of the antibody as observed in the albumin antigen assay is in good agreement with the results of other researchers (Alvarez, et al., 1988; Rodwell, et al., 1986). A more definitive assay system, such as a competitive binding format would be useful to clarify these observations, but was unavailable at the time.

Table 4.25 ELISA assay of PDTA-MAb 155H.7 conjugates

Conjugates (μg)	M1[a]	M2[b]	M3[c]	Native MAb
		(Absorbance at 410 nm)[d]		
	LS174T as the antigen			
10	0.70 (0.04)	0.66 (0.07)	0.68 (0.0[illegible])	0.48 (0.06)
5	0.69 (0.07)	0.66 (0.06)	0.70 (0.04)	0.40 (0.06)
2.5	0.66 (0.06)	0.65 (0.07)	0.68 (0.08)	0.31 (0.04)
1	0.66 (0.06)	0.59 (0.07)	0.64 (0.04)	0.15 (0.03)
0.1	0.40 (0.03)	0.24 (0.03)	0.14 (0.02)	0.11 (0.06)
	HSA as the antigen			
5	0.60 (0.05)	0.62 (0.03)	0.62 (0.05)	0.53 (0.05)
2.5	0.60 (0.06)	0.54 (0.07)	0.58 (0.07)	0.48 (0.03)
0.1	0.29 (0.06)	0.26 (0.07)	0.17 (0.04)	0.22 (0.03)

a: One step reaction with 30 mM of $NaIO_4$; a starting ratio of chelate to MAb 155H.7 of 20:1; reaction for 1 hour at 4°C

b: The reaction conditions were the same as those in (a) except for a reaction time of 30 minutes

c: Oxidation with 30 mM of $NaIO_4$; reaction at 28°C for 30 minutes. Reduction conjugation at a starting ratio of chelate to MAb 155H.7 of 20:1 and 10 mM of $NaBH_3CN$ for 12 hours

d: S.D. in parentheses. n=3

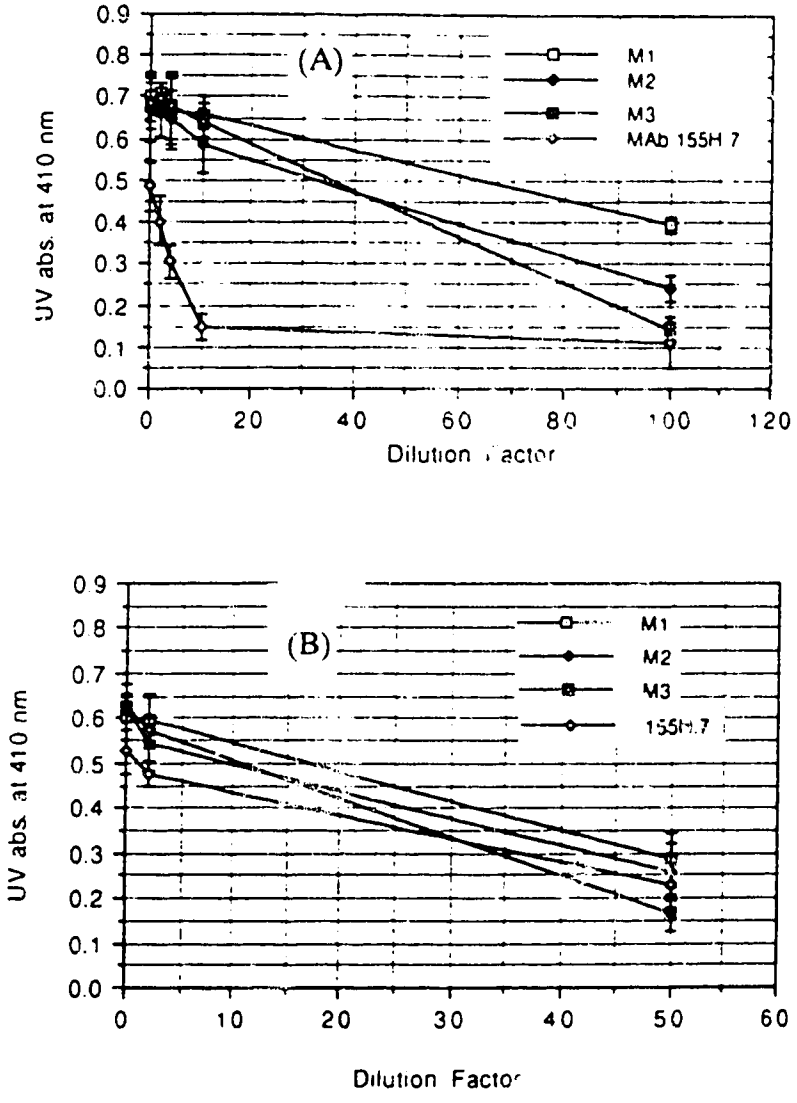


Fig. 4.28 ELISA assay of PDTA-MAb 155. .7 conjugate. A: LS174T as the antigen. B: HSA as the antigen. M1, M2 and M3 are the same as those in Table 4.25

4.11.8 Native PAGE Assay

Biochemical changes on modified antibodies such as the charge and the formation of oligomers, were also examined by native PAGE. Since native PAGE separates components on the basis of their size, net charge and conformation, the increase in the net negative charge by linking PDTA to the antibody molecule should result in an increase in electrophoretic mobility. Indeed, a pattern was observed in which all the PDTA conjugates showed a greater migration towards the anode than those of native antibodies (Fig. 4.29). There were no additional bands of PDTA-hIgG and PDTA-MAb 155H.7 conjugates observed, indicating the absence of the oligomers including those held together by non-covalent binding.

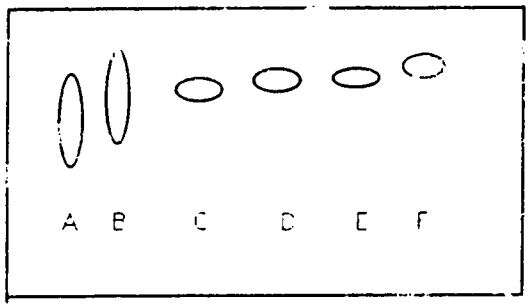


Fig. 4.29 Native PAGE assay of PDTA-antibody conjugate

4.11.9 Biodistribution of PDTA-antibody Conjugates in Normal Mice

Results from the tissue biodistribution of ^{111}In-labeled PDTA-antibody conjugates in normal ICR mice at 24 and 48 hours after iv injection are summarized in Table 4.26. We compared two preparations of hIgG conjugates in which PAB-PDTA was conjugated to oxidized carbohydrate residues then subjected to reduction of the Schiff's base (A) as well as hIgG conjugate prepared by the one-step process

(B) which did not involve any reduction. Results indicated that preparation A had a whole body radioactivity of 82% and 74.9% after 24 and 48 hours respectively, while preparation B had a slightly faster clearance, with a retained whole body radioactivity of 73.4% and 58.3% for the same time periods. One of the reasons for the slightly faster clearance of the preparation B might be attributed to it being less stable *in vivo* as a result of the elimination of the reduction step in the conjugation reaction. However although this difference is statistically significant ($p<0.05$) the magnitude of the difference is very small (about 5%).

Laguzza, et al., (1989) reported in utilizing the hydrolysis of hydrazone bond, consequently releasing the free hydrazide compound which was an antitumor drug at acidic pH at tumor sites. Their data showed that about 8% of the free drug, hydrazide was released *in vitro* when incubated in PBS buffer at pH 7.4 at 37°C for 24 hours. While release became more significant at pH 5.3 at 37°C, with about 28% after 24 hours incubation. Further investigation for *in vitro* stability of the PDTA-conjugate at different pH and *in vivo* distribution in tumor-bearing mice would be needed to assess the influence of a possible cleavable linkage between chelate and antibodies on reduction of the non-target background.

Liver uptake was in the range of 4 to 6% of the injected dose/g of tissue, which also agrees very well with those previously observed (Table 4.2) in section 4.3 that there were no observable aggregate or oligomer formation during the modification of the antibodies. In the case of the reduction preparation, the accumulation appears to be higher in the liver than that of one step preparation (5.95%, 5.09% *vs* 4.38% and 4.33%) at 24 and 48 hours PI. Less manipulation of preinjected samples may account for the less uptake in the liver in this case, but again the magnitude of the difference (<1%) makes conclusive statement difficult but we believe the values are comparable. The high kidney uptake indicates that this organ appears to be the main site of excretion of ^{111}In-labeled PDTA-antibody conjugate in mice. The benefit of a fast clearance from the circulation is a lower background and possibly a higher tumor to background ratio as long as a reasonable uptake in tumor is achieved.

Table 4.26 Biodistribution of ^{111}In labeled PDTA-hIgG conjugate in normal ICR mice[a]

Tissues	Reduction process[b]		One step process[c]	
	24 hr	48 hr	24 hr	48 hr
Blood	1.17(0.10)	0.37(0.03)	0.95(0.39)	0.40(0.33)
Liver	5.95(0.33)	5.09(0.48)	4.38(0.44)	4.13(0.31)
lungs	2.86(0.19)	1.97(0.14)	2.13(0.73)	2.45(0.76)
Kidney	20.29(0.52)	14.27(1.17)	21.69(3.26)	13.3(1.88)
Spleen	5.53(0.42)	3.98(0.65)	3.70(1.03)	3.04(0.59)
Bone	5.25(0.34)	4.97(0.31)	4.09(1.31)	3.85(0.64)
Muscle	1.81(0.11)	1.49(0.13)	1.13(0.25)	1.41(0.35)
Whole body Remaining	82.8(2.8)	74.9(2.5)	73.8(4.1)	68.3(4.3)

a: % injected dose/g. S.D. in parentheses. n=3

b: About 4.5 PDTA residues per hIgG molecule

c: About 1 PDTA residue per hIgG molecule

Tissue biodistribution results of ^{111}In-PDTA-MAb155H.7 and ^{111}In-EDTA-MAb155H.7 are tabulated in Table 4.27., which shows a very similar overall pattern of uptake for both chelated forms of this antibody. This reaffirms the in vivo stability of the linking process to the oxidized antibody since it behaves analogously to that prepared via the bromoacetamido linkage reaction. The most notable difference occurs in the blood which may represent small changes in the vascular permeability or metabolism of these conjugates due to the site of chelate attachment. The value for kidney uptake of 20.19%/g and 19.78%/g is shown

Table 4.27 Biodistribution of ^{111}In-PDTA-MAb155H.7 and ^{111}In-EDTA-MAb155H.7 in normal ICR mice 24 hours PI[a]

Tissues	^{111}In-PDTA-MAb	^{111}In-EDTA-MAb[b]
Blood	0.90(0.10)	5.5(0.53)
Liver	4.23(0.45)	5.81(0.59)
Lungs	2.03(0.34)	3.96(0.38)
Kidneys	19.78(1.17)	20.9(4.11)
Spleen	3.93(0.46)	5.75(0.75)
Bone	3.30(0.96)	3.27(0.44)
Muscle	1.91(0.34)	0.60(0.10)

a: % dose inj./g with S.D. in parentheses

b: Data from Turner, et al., 1988

for ^{111}In-EDTA-MAb and ^{111}In-PDTA-MAb respectively. The slow decrease in kidney after 48 hours PI (Table 4.26) could not be attributed solely to the excretion of the metabolized chelate. Some kind of uptake mechanism may be present for the ^{111}In-labeled MAb155H.7. Clearly, further studies on the physiological behaviour of chelate-antibody conjugates prepared by different modification processes is warranted.

5. SUMMARY AND CONCLUSIONS

Based on the initial objectives of this work, as stated in the introduction, the following summary and conclusions can be made:

1 a. GalNAc and GalGalNAc-hIgG conjugates were prepared *via* a novel fluorinated linking arm, HFGDE, reacting with amino derivatives of the sugars. The number of sugar residues on conjugates was controlled by varying starting ratio of sugars to antibodies.

b. Estimation of the sugar residues on antibody conjugates by ^{19}F-NMR signals from fluorinated linking arm provided an useful tool and an alternative to the conventional phenol sulfuric acid method.

c. A 2.1 hour half life of hydrolysis of methyl ester bond in PDT linked fluorinated arm (HFGDE, Fig. 3.2) at 20°C was obtained by analyzing changes of the methyl proton signals in ^{1}H-NMR spectrum.

d. Sialic acid-hIgG conjugate was prepared by reductive amination of an aldehyde derivative of sialic acid with the side chain amino groups on hIgG molecule. Fifteen residues were attached per hIgG molecule after a 60 hour reaction as analyzed by the resorcinol method.

e. Biodistribution studies of radioiodinated sugar-hIgG conjugates in normal ICR mice showed high liver uptake and low blood level for both GalNAc and GalGalNAc conjugates when compared to the radioiodinated native antibody 15 minutes PI, suggesting uptake of the radioiodinated sugar conjugates probably *via* the hepatic receptor in the liver. There was no significant difference observed in tissue biodistributions between native hIgG and a non-sugar amino compound-hIgG conjugate *via* the fluorinated linking arm indicated that the changes in biodistribution was caused by carbohydrates but not fluorinated linking arm itself. The technique can be applied to MAbs to selectively modify their biological behaviour *in vivo*.

f. There were no significant differences observed between radioiodinated sialic acid-hIgG conjugate and native hIgG in liver uptake and blood level in

normal ICR mice. The results indicated that conjugation of sialic acid to the amino acid backbone which introduced more negative charge did not have any influence on the liver uptake and blood clearance even though the protecting effect of terminal sialic acids on carbohydrate chains of glycoproteins was partly attributed to the existence of the negative charge on the sialic acid molecule.

2 a. Antibodies, hIgG and MAb155H.7 were chemically modified on their oligosaccharide moieties by destroying terminal, and some of the innerchain sugars with the formation of a -CHOH group, addition of an amino acid (tyrosine hydrazide), and conjugation with a lipophilic compound, DNPH.

b. Studies on periodate oxidation of antibodies and a neoglycoprotein (HSA-Tα) and subsequent conjugation of the oxidized antibodies with DNPH suggested the reproducibility and the specificity of the method mainly to the carbohydrate moieties of antibodies. The method may be used in measuring sugar content of simple sugar-protein conjugates. The aggregate formation study of oxidized antibodies during storage indicate that there is no intermolecular condensation oberved, but the aldehyde groups produced by periodate oxidation may be lost gradually probably by intramolecular condensation, though the direct evidence is lacking in this regard.

c. Biodistribution of the radioiodinated antibodies modified on their carbohydrate moieties showed that the above modifications of carbohydrate chains in Fc portion of the antibodies did not have apparent influence on their tissue distribution in mice at least at an early stage. The results also suggested that the asialoglycoprotein receptor may not play an important role in the clearance of hIgG molecules in mice.

3 a. PAB-PDTA was conjugated to hIgG and MAb155H.7 by periodate oxidation of oligosaccharide moieties of antibodies and then reduction of Schiff base with $NaCNBH_3$. Approximately five PDTA molecules were coupled to the antibody molecule. The reduction step may be eliminated when aromatic amino compound such as PAB-PDTA are used. A one step conjugation in

which PAB-PDTA, $NaIO_4$ and antibody were simply added together was developed. Approximately one chelate was coupled onto each antibody molecule by this one step conjugation method.

b. There were no significant changes observed in immunoreactivity of PDTA-MAb155H.7 conjugates by modification of carbohydrate moieties of the antibody analyzed by ELISA assay although a suspended increase in non-specific binding was observed.

c. One step conjugation *via* carbohydrate moiety of hIgG and MAb155H.7 appeared to be a useful method in modification of antibodies because of it's simplicity, mild reaction conditions, and reasonable amount of conjugation of aromatic amino and hydrazine compounds to antibodies.

d. PDTA-hIgG conjugates were radiolabeled with ^{111}In-citrate with 95% protein bound radioactivity assayed by ITLC. A similar results were obtained when HPLC 3000SW size exclusion column and a Sephadex G-50 column were used in analysis.

e. Direct binding of ^{111}In-citrate to oxidized antibodies was observed when TLC chromatography were applied. This non-specifically bound ^{111}In as observed by TLC survived a challenge of up to 50 fold excess free chelates, PAB-PDTA and EDTA. However, separation by Sephadex G-50 gel chromatography showed this to be non-specific binding.

f. Biodistribution of PDTA-antibody conjugates in normal ICR mice was consistent with previousiy reported data using a different chelating agent of the same class.

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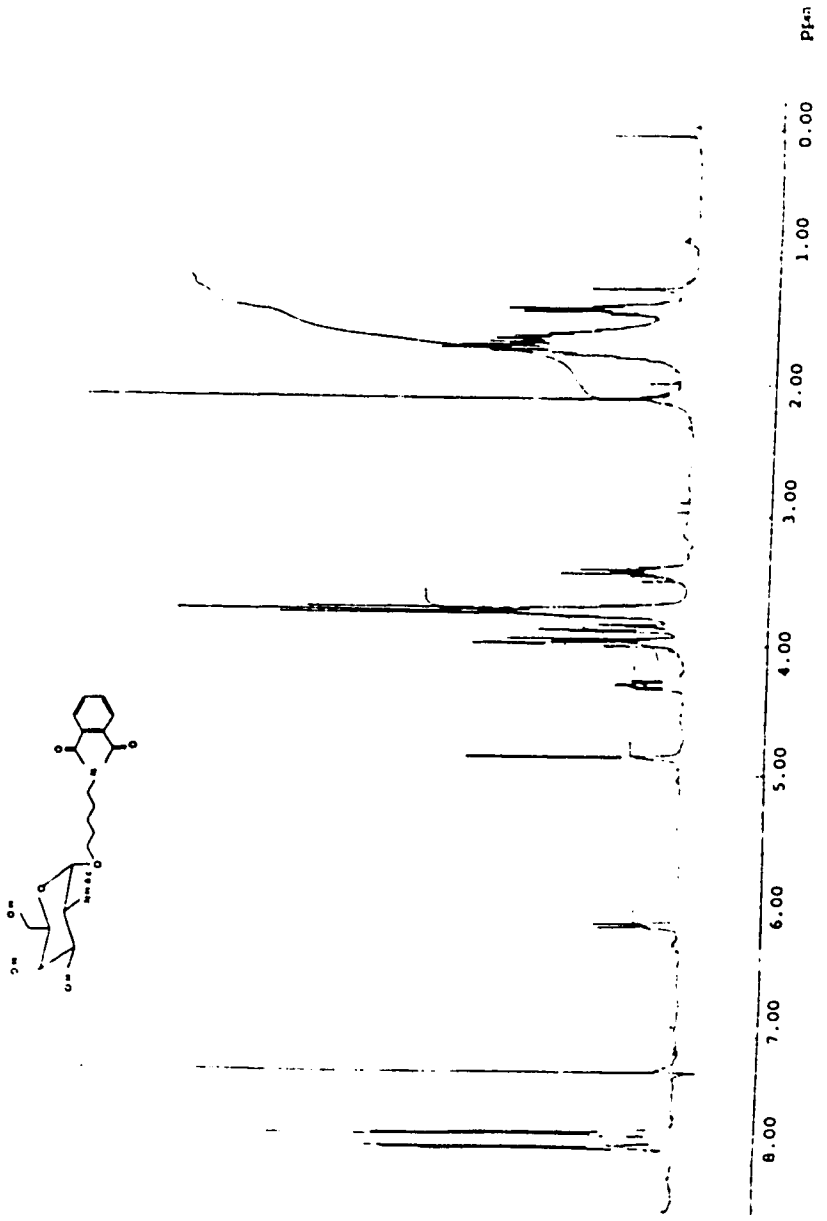
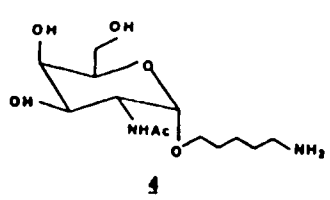
7. APPENDICES

Appendix 1 ^{1}H-NMR spectrum of compound 1 (Fig. 3.1)

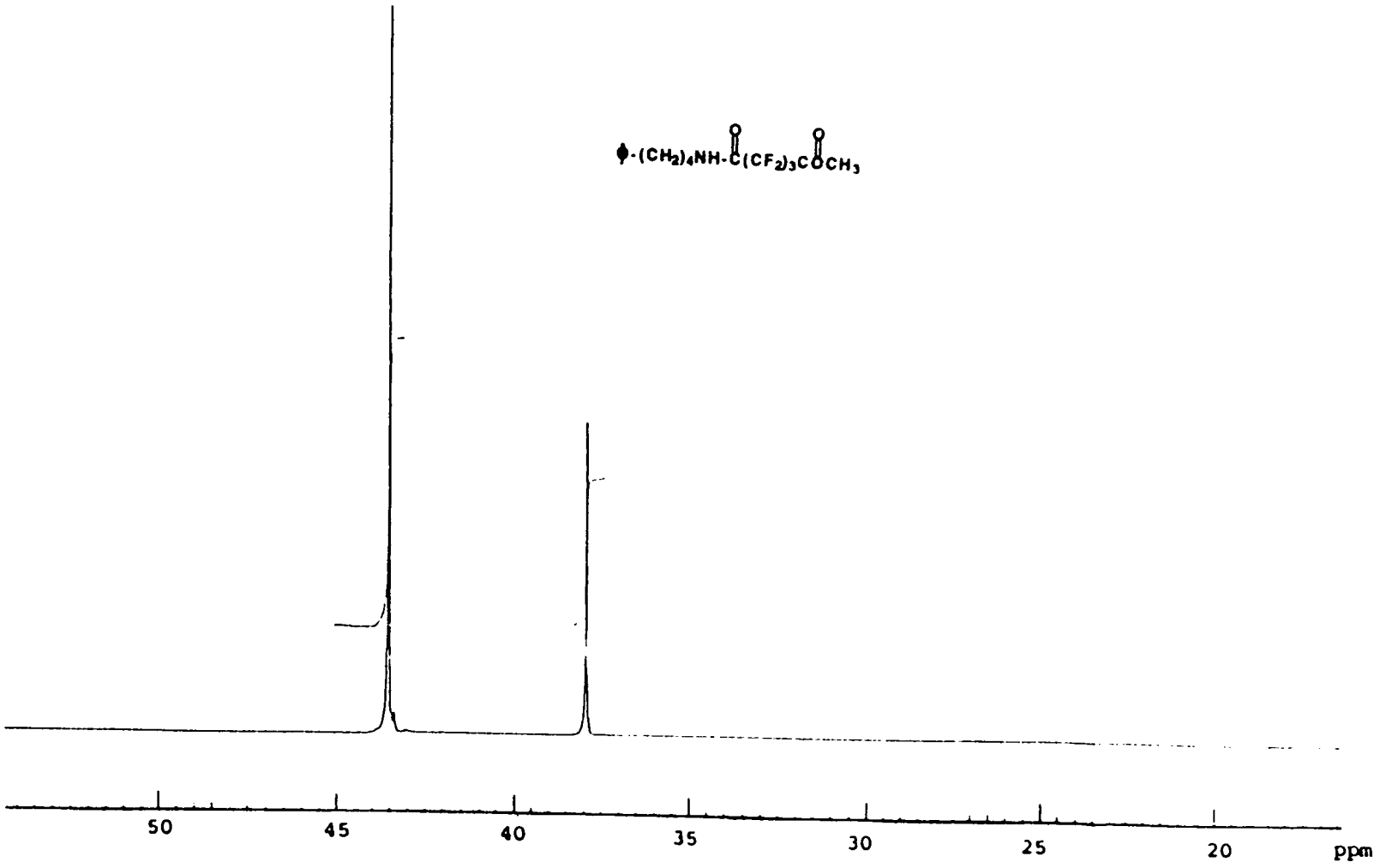


Appendix 2 ^{1}H-NMR spectrum of compound **4** (Fig. 3.1)

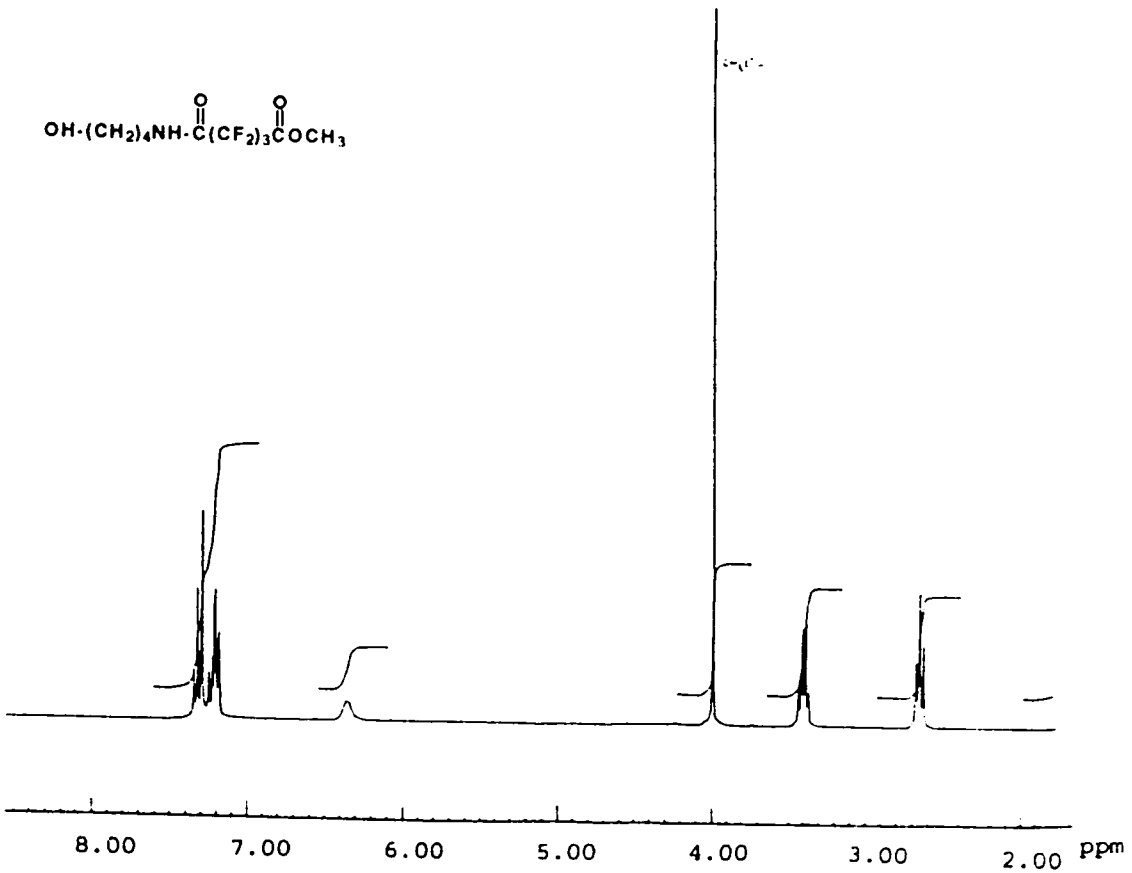


4

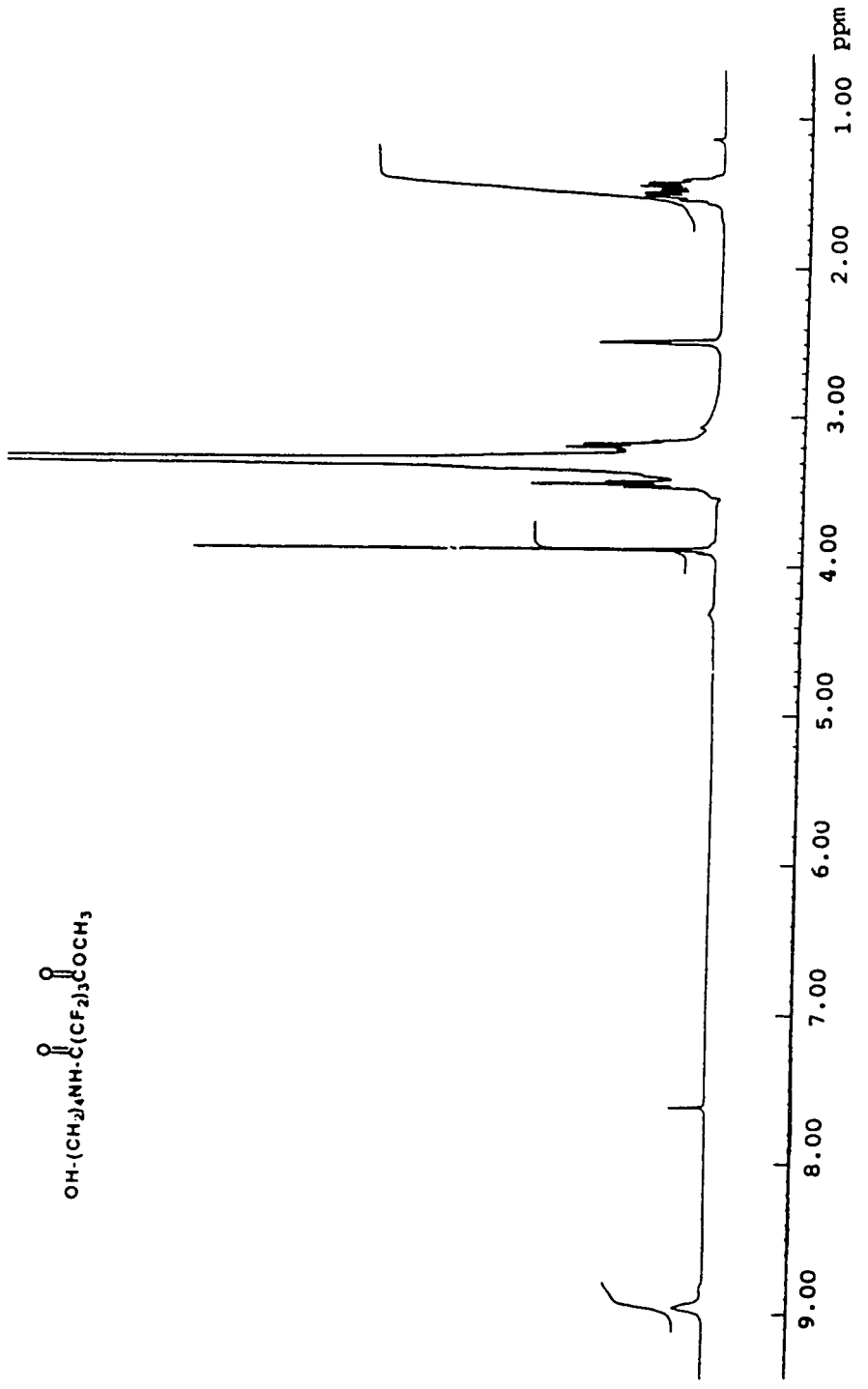
Appendix 3 ^{19}F-NMR spectrum of compound **5**, HFGDE, (Fig. 3.2)



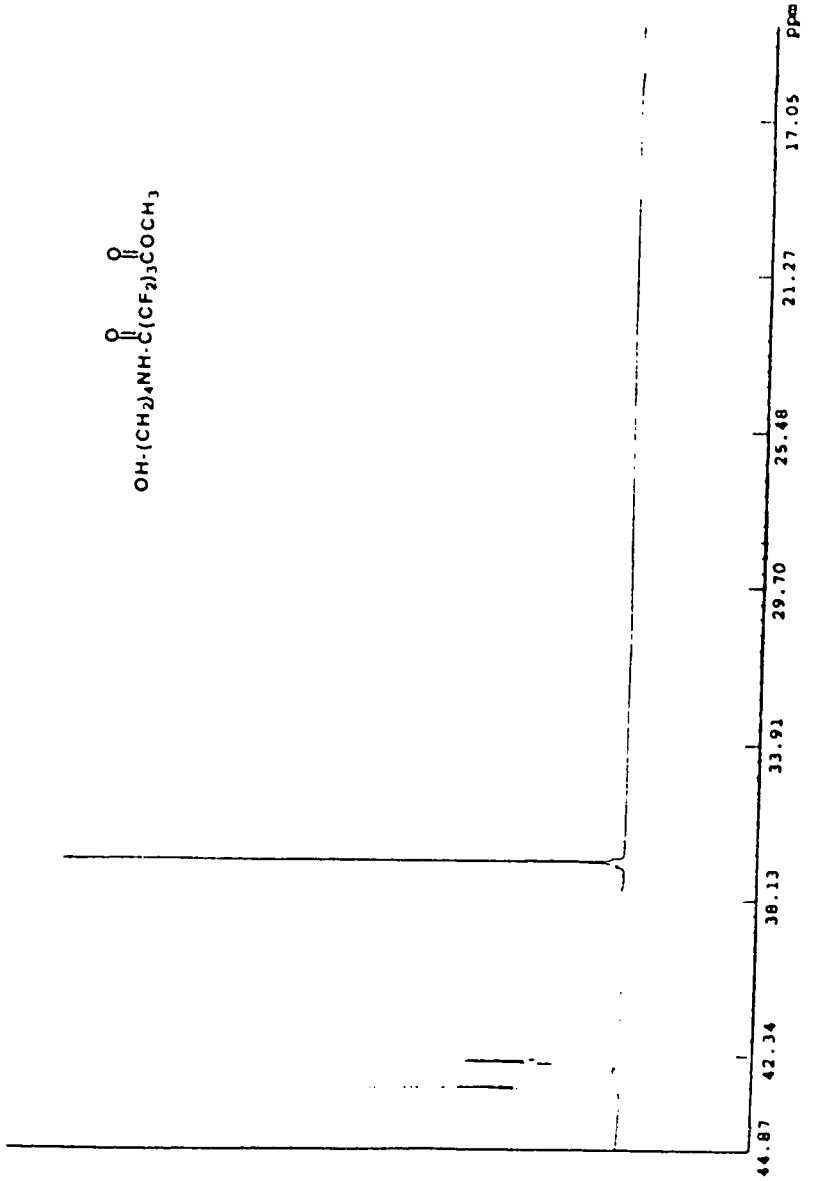
Appendix 4 ^{1}H-NMR spectrum of compound 7c (Fig. 3.2)



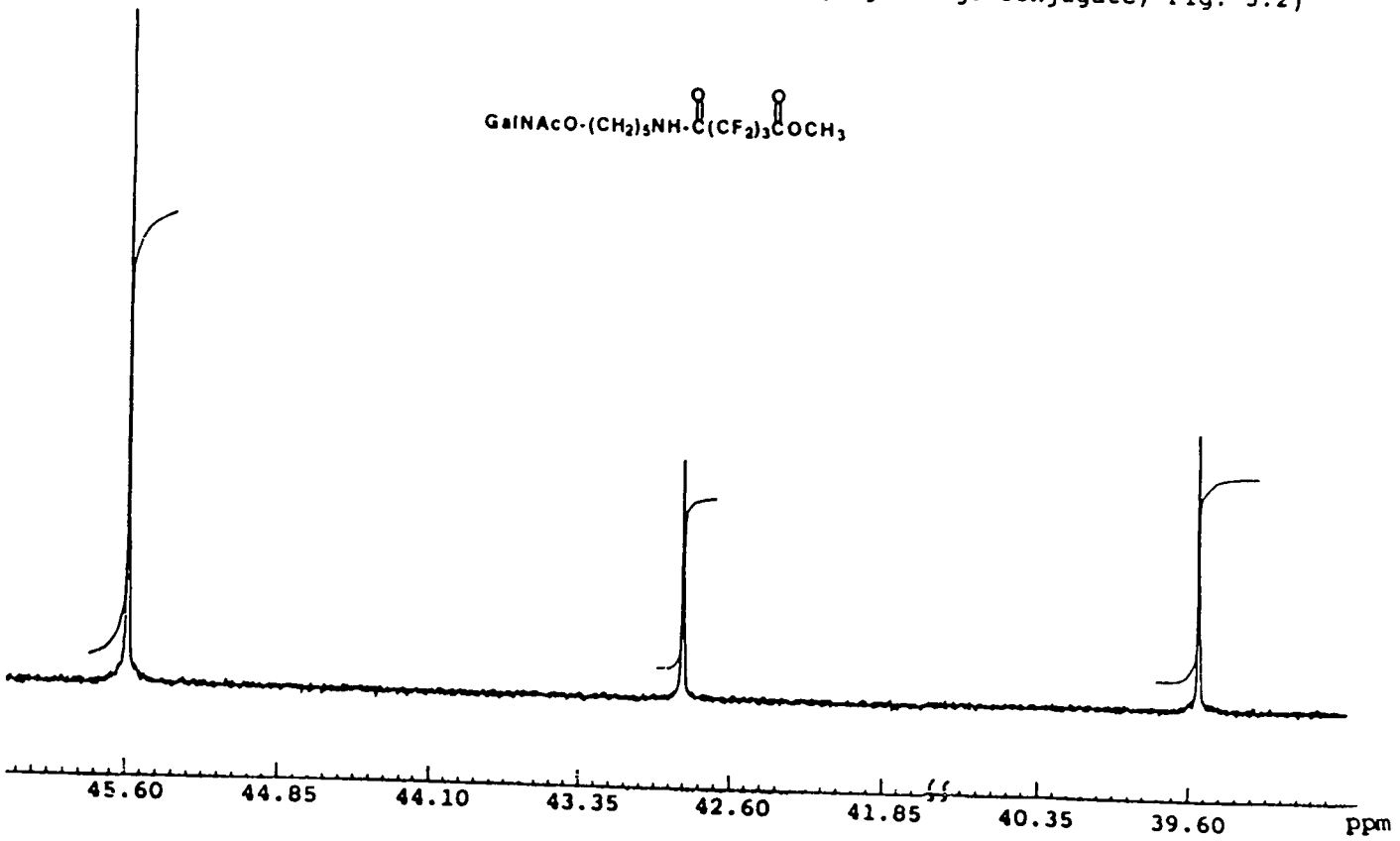
Appendix 5 ^{1}H-NMR spectrum of compound 7d (Fig. 3.2)



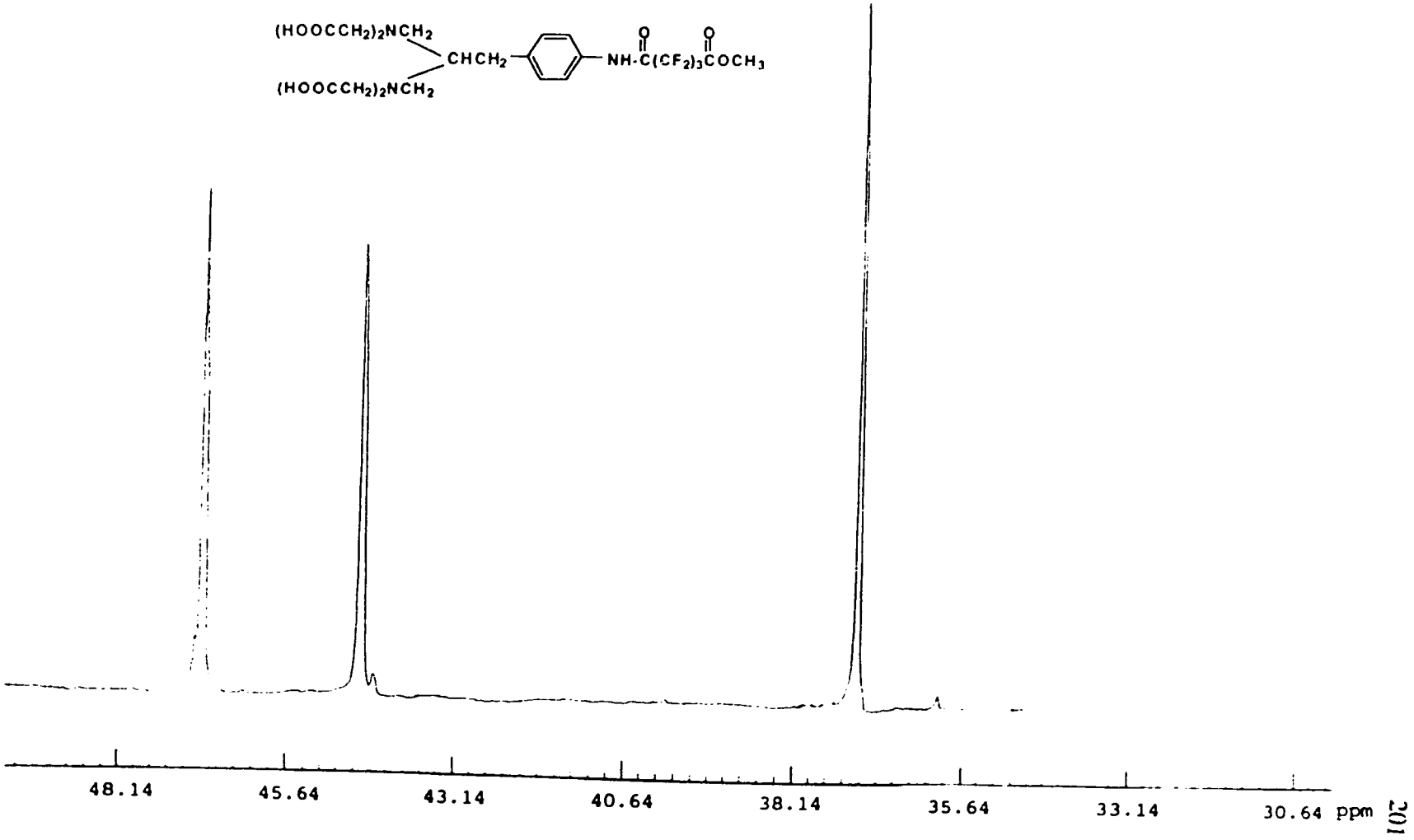
Appendix 6 ^{19}F-NMR spectrum of compound 7d, (Fig. 3.2)



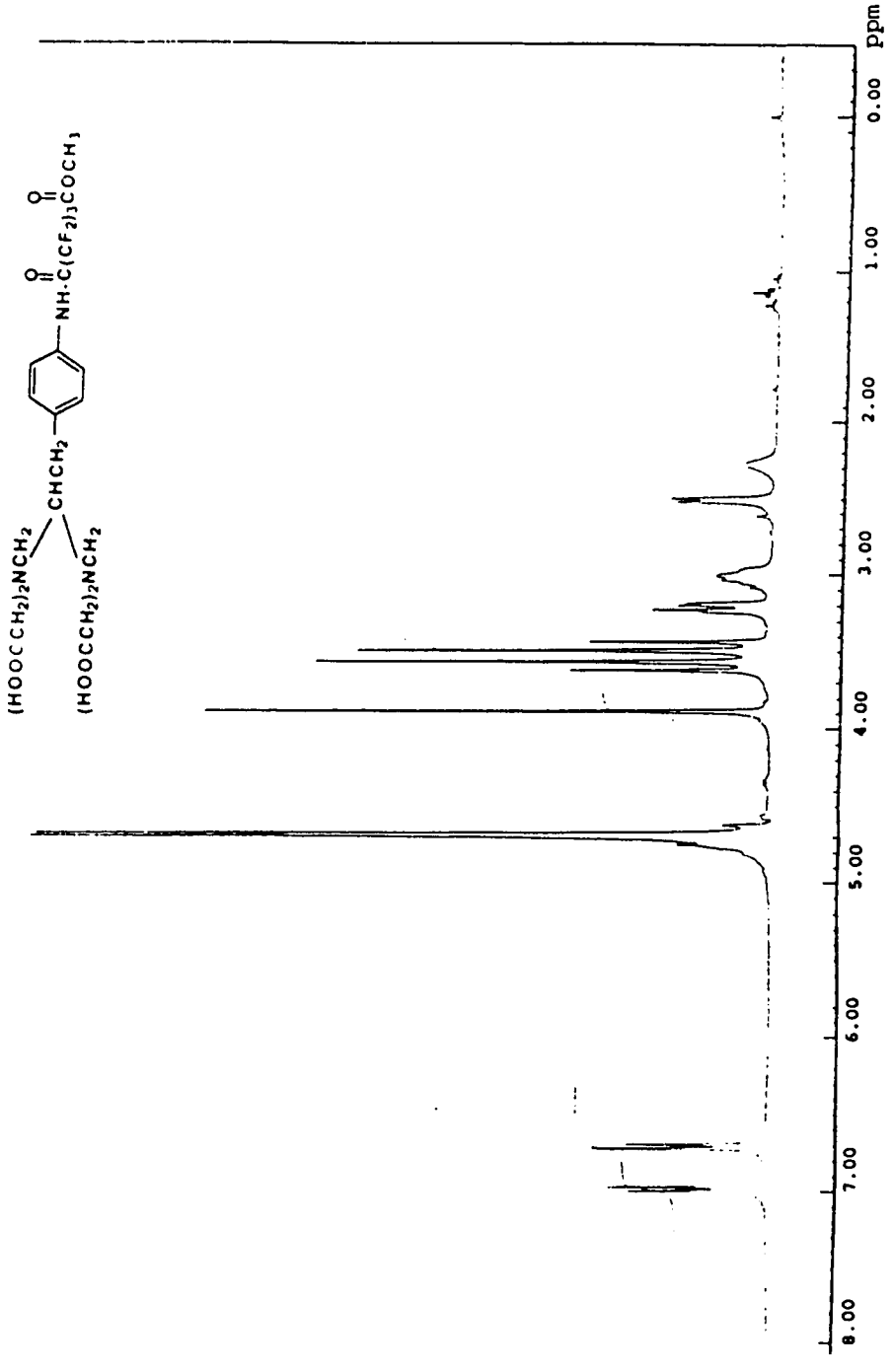
Appendix 7 ^{19}F-NMR spectrum of compound **8a**, (sugar-hIgG conjugate, Fig. 3.2)



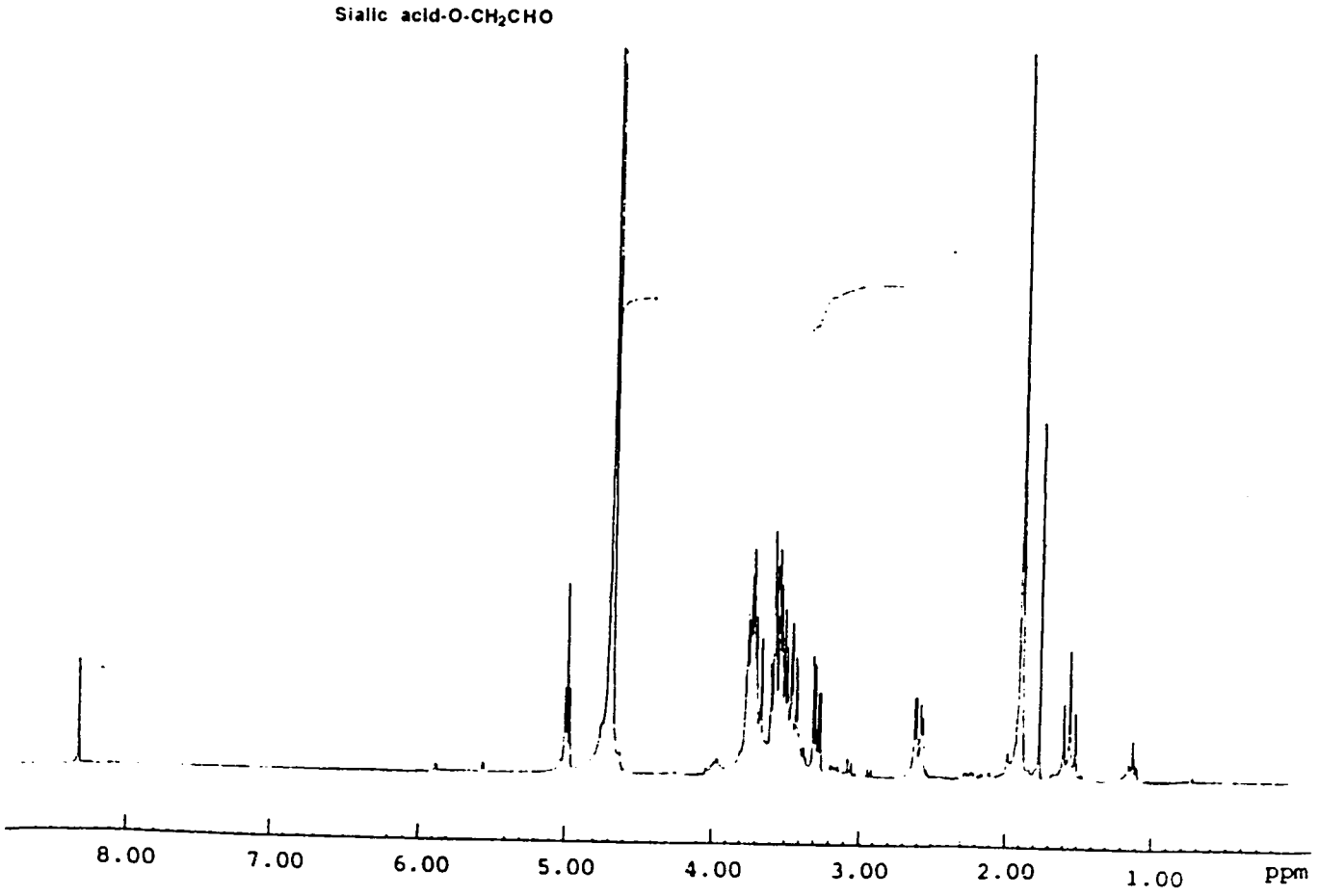
Appendix 8 ^{19}F-NMR spectrum of compound 7e, (Fig. 3.2)



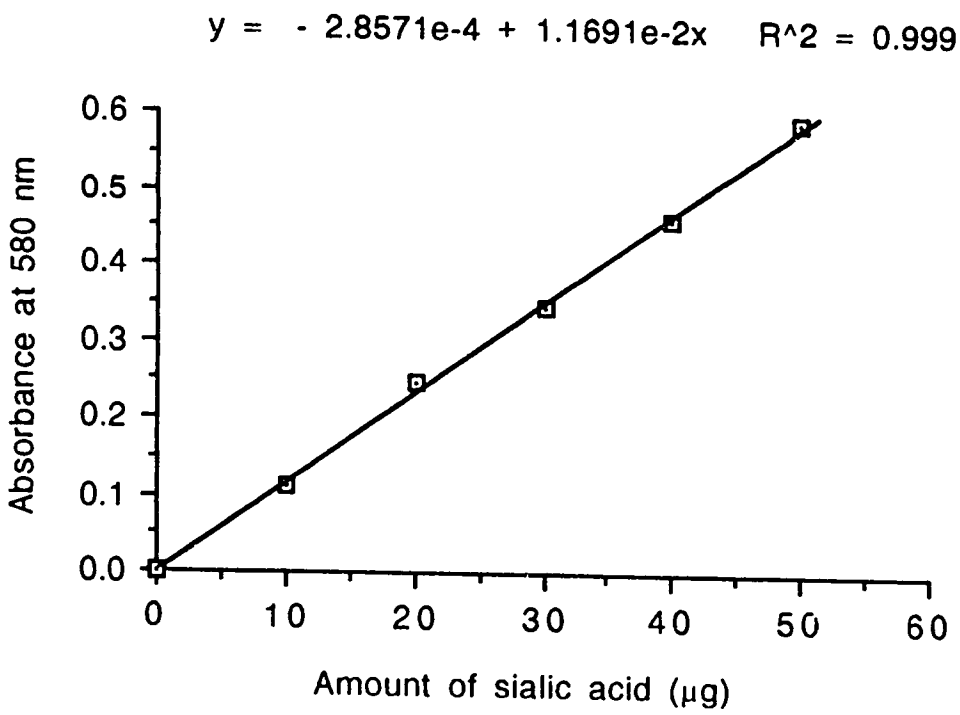
Appendix 9 ^{1}H-NMR spectrum of compound 7e (Fig. 3.2)



Appendix 10 ^{1}H-NMR spectrum of compound 10 (Fig. 3.3)



Appendix 11 Sialic acid standard curve assayed by resorcinol method



Appendix 12 ^{1}H-NMR spectrum of compound **6e** (Fig. 3.2)