CANADIAN THESES

THÈSES CANADIENNES

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30.

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30.

THIS DISSERTATION HAS BEEN MICROFILMED EXACTLY AS RECEIVED

LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS REÇUE

THE UNIVERSITY OF ALBERTA

A Comparative Study of ~~Radiochlo~~lated and Radioiodinated Monoclonal Antibodies

by

Cornelia J. Turner

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Master of Science

IN

Pharmaceutical Sciences (Bionucleonics)

Faculty of Pharmacy and Pharmaceutical Sciences

EDMONTON, ALBERTA

Fall 1986

ISBN 0-315-32425-2

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR Cornelia J. Turner

TITLE OF THESIS A Comparative Study of Radiochelated and Radioiodinated Monoclonal Antibodies

DEGREE FOR WHICH THESIS WAS PRESENTED Master of Science

YEAR THIS DEGREE GRANTED Fall 1986

Permission is hereby granted to THE UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(SIGNED) .....

PERMANENT ADDRESS:

.....

.....

.....

DATED 19

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled A Comparative Study of Radiochelated and Radioiodinated Monoclonal Antibodies submitted by Cornelia J. Turner in partial fulfilment of the requirements for the degree of Master of Science in Pharmaceutical Sciences (Bionucleonics).

.....

Supervisor

.....

.....

.....

.....

Date.....

To Mom,

for all her love and understanding,

and to the loving memory

of Dad

ABSTRACT

The use of radiolabeled monoclonal antibodies (MAbs) for *in vivo* cancer detection has been under investigation for over a decade. Pioneering studies involved the use of radioiodine and in particular, ^{131}I. The many disadvantages of the radioiodine isotopes, such as *in vivo* thyroid accumulation of radioiodide and the less than optimal imaging characteristics prompted the search for more suitable radioisotopes and methods to incorporate these radioisotopes into MAbs. Development of bifunctional chelating agents, such as DTPA anhydride and derivatized benzyl-EDTA, allowed the use of radioisotopes such as ^{111}In, ^{67}Ga and ^{99m}Tc.

For the studies presented here, two derivatives of benzyl-EDTA, p-benzyldiazonium-EDTA and bromoacetamido-benzyl-EDTA, were investigated for their ability to label proteins and in particular, MAbs. Preliminary studies with thin layer chromatography analysis (TLC) demonstrated that the bifunctional chelate could bind ^{111}In and ^{67}Ga. Purity of the radiometal solutions was assessed using TLC and instrumental neutron activation analysis (INAA). The stability of the ^{111}In-bifunctional chelate was determined in the presence of transferrin *in vitro* using gel filtration techniques.

The two derivatives were investigated under varying reaction conditions for their ability to label human serum albumin (HSA), polyclonal human IgG (poly-H-IgG), MAb-46D, MAb-M1A and MAb-155H.7. The bromoacetamido derivative (BrAc-B-EDTA) was superior to the p-benzyldiazonium derivative (N$_2^+$-B-EDTA) and was used for comparative studies between radioiodinated and radiochelated MAb-155H.7. Three different iodinating reagents were assessed for their effects on *in vitro* MAb function using MAb-155H.7. Both conventional gel filtration techniques and a centrifuged mini-column gel filtration technique as well as TLC were used for analysis of radioiodinated and radiochelated MAb.

In vitro assessment of radioiodinated and radiochelated MAb-155H.7 was conducted using an enzyme-linked immunosorbent assay (ELISA), a whole cell ELISA, live cell uptake on murine tumour cells, a radioactive binding assay (RBA) and histological screening on human adenocarcinoma tissue sections. For MAb-155H.7, the ELISA using the immunizing

antigen gave the best indication of the effects of the various labeling techniques on the *in vitro* function of the MAb.

In vivo assessment included preliminary biodistributions of $Na^{123}I$ and ^{111}In-citrate, ^{125}I-p-aminobenzyl-EDTA-^{111}In and ^{125}I-poly-H-IgG and ^{111}In-B-EDTA-poly-H-IgG in normal Balb/C mice. As well, the biodistributions of ^{125}I-poly-H-IgG, ^{111}In-B-EDTA-MAb-155H.7 and ^{131}I-MAb-155H.7 were compared in TA3/Ha tumour bearing mice. [redacted] studies showed that the ^{111}In-p-aminobenzyl-EDTA was very stable in the presence of transferrin and that [redacted] EDTA-MAb-155H.7 was superior to ^{131}I-MAb-155H.7 and ^{125}I-poly-H-IgG for TA3/Ha tumour accumulation. Radionuclide scans of TA3/Ha tumour bearing mice also demonstrated visually the superiority of the ^{111}In-B-EDTA-MAb-155H.7 for tumour accumulation.

Bromoacetamido-benzyl-EDTA has proven to be a useful compound for the incorporation of radioisotopes other than radioiodine into MAbs. Although *in vitro* testing showed little difference between radioiodinated and radiochelated MAb, the *in vivo* distributions in TA3/Ha tumour bearing mice demonstrated the superiority of the ^{111}In bifunctional chelate label for tumour accumulation.

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to Dr. A.A. Noujaim for his guidance during the course of this research and his friendship over the years.

Special thanks go to Rene Gaudreault for the synthesis of the bifunctional chelating agent.

I would also like to thank Dr. B.M. Longenecker, Mary Jane Meeker, Lindsay MacQueen and Bobina Widtman of the Department of Immunology, U of A for their help and advice with the tumour cell studies and the animal work; Dr. D. Willans and Grazyna Stanczyk-Brzezinska of the Edmonton General Hospital for help with the histology work; Chris Ediss, Steve MacQuarrie and Richard Flanagan of the Faculty of Pharmacy, U of A for their expert advice and technical assistance; Dennis Ng, Pete Ford and John Duke of the U of A SLOWPOKE Reactor Facility for their help with the activation analysis and Dr. A. Shysh for many helpful discussions.

Sincere thanks and appreciation are extended to Dr. D. Lyster and Terry Rihela of Vancouver General Hospital and thanks to Dr. J. Levy and Daphne Mew of the Department of Microbiology, UBC for their help with the MAb-M1A and MAb-46D experiments.

The support and friendship of the students, staff and associates of the Division of Bionucleonics and Radiopharmacy, especially John, Kerry, Pam, Gilbert, Diane, Tony, Anneke and Uwe, is greatly appreciated.

My deepest gratitude is reserved for Tom, for his continuous spiritual support and timely scientific advice, and without whom this chapter in my life could not have been written.

The financial support from an Alberta Heritage Foundation for Medical Research studentship and from a Warner-Lambert/Parke-Davis Research scholarship is gratefully acknowledged.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

CEA	- carcinoembryonic antigen
AFP	- alphafetoprotein
HCG	- human chorionic gonadotropin
MAb	- monoclonal antibody
HPRT	- hypoxanthine phosphoribosyl transferase
PEG	- polyethylene glycol
HAT	- hypoxanthine, aminopterin, thymidine
ELISA	- enzyme-linked immunosorbent assay
RBA	- radioactive binding assay
RIA	- radioimmunoassay
EBV	- Epstein Barr virus
INAA	- instrumental neutron activation analysis
TLC	- thin layer chromatography
TCA	- trichloroacetic acid
ICl	- iodine monochloride
Cl-T	- chloramine-T
Iodo-gen	- 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril
EDTA	- ethylenediaminetetraacetic acid
DTPA	- diethylenetriaminepentaacetic acid
B-EDTA	- benzyl-ethylenediaminetetraacetic acid
DTPAan	- diethylenetriaminepentaacetic acid anhydride
DTPAnhsucester	- DTPA-N-hydroxysuccinimide ester
PNB-EDTA	- p-nitrobenzyl-ethylenediaminetetraacetic acid
PAB-EDTA	- p-aminobenzyl-ethylenediaminetetraaceitc acid
N;-B-EDTA	- diazobenzyl-ethylenediaminetetraacetic acid
BrAc-B-EDTA	- bromoacetamido-benzyl-ethylenediaminetetraacetic acid

AcCN	- acetonitrile
AmAc	- ammonium acetate
MeOH	- methanol
PEI	- polyethylimine
HSA	- human serum albumin
BSA	- bovine serum albumin
KLH	- keyhole limpet hemocyanin
FBS	- fetal bovine serum
PBS	- phosphate buffered saline
HRPO	- horseradish peroxidase
AP	- alkaline phosphatase
ABTS	- 2,2'-azino-di(3-ethylbenzthiazoline sulphonate)
pNPP	- p-nitrophenylphosphate
ABC reagent	- avidin-biotin HRPO (Vectastain)
DAB	- diaminobenzidine tetrahydrochloride
Tβ-S	- Tβ-synsorb (Chembiomed)
Tα-S	- Tα-synsorb (Chembiomed)
B-S	- B-synsorb (Chembiomed)

1. SURVEY OF THE LITERATURE

1.1 INTRODUCTION

The concept of using antibodies for the diagnosis and treatment of cancer has intrigued scientists for decades. The first reported use of anti-sera for therapy of cancer was described by Hericourt and Richet in 1895.[1,2] In 1953 and 1954 Pressman and Korngold [3-5] demonstrated the ability of radiolabeled polyclonal antibodies against rodent tumours to localize specifically in transplanted animal tumours. A few years later, the same group proved the diagnostic usefulness of ^{131}I-labeled polyclonal antibodies by demonstrating their localization in animal tumours using external scintigraphy.[6] In 1960, Bale *et al.*[7] succeeded in showing that polyclonal anti-tumour antibodies could carry toxic doses of radionuclides to animal tumours. The first human trial of radiolabeled polyclonal antibody was performed in 1974 using ^{131}I-anti-carcinoembryonic antigen (^{131}I-anti-CEA).[13] Since that first human study the use of radiolabeled polyclonal and monoclonal antibodies for diagnosis and therapy of cancer has expanded dramatically.[11,14,15]

In initial experiments polyclonal anti-sera were produced by immunizing the appropriate animal with whole tumour cells or cell fragments. Tumour specific antigens had not been identified and the resulting antibody preparation showed a high degree of reactivity with normal tissues when used *in vivo*. Purification techniques such as tissue absorption and affinity chromatography improved the quality of these preparations but the final result was still disappointing.

The search for tumour specific antigens has not been successful since many of the antigens that were once thought to be tumour specific are only tumour-associated as they have also been discovered in some normal adult or fetal tissue. The presence of these antigens is merely elevated in cancer or the antigens may be expressed as incomplete synthetic products of the cancer cells.[8-10] Among these are the oncofetal antigens, such as alphafetoprotein (AFP) and carcinoembryonic antigen (CEA).[9] Other tumour-associated antigens can be normal constituents of cells, such as prostatic acid phosphatase, insulin, ferritin and human

chorionic gonadotropin (βHCG).[9] More recently, tumour-associated antigens residing in tumour cell membranes have been identified and among these are the melanoma p97 antigen[11] and the Thomsen-Friedenreich antigen (T-antigen) expressed in carcinomas.[12]

1.2 MONOCLONAL ANTIBODIES

The rapid expansion of both the identification of tumour-associated antigens and the application of antibodies to *in vivo* diagnosis and therapy can be attributed largely to the development of monoclonal antibodies (MAb). In 1975, Kohler and Milstein[14] developed the hybridoma technology that allows the production of these highly specific MAbs. The original technique has been refined and today a variety of mouse, rat and human MAbs have been generated. The basic hybridoma technique consists of the fusion of a sensitized B lymphocyte with a stable, non immunoglobulin producing murine myeloma cell line. Sensitization is usually accomplished by immunizing a mouse with the appropriate antigen and, depending upon the protocol, the animal is sacrificed from 4 weeks to 4 months after beginning the immunizing schedule. The spleen is a convenient source of large numbers of B lymphocytes and a suspension of the sensitized spleen cells is fused with the myeloma cells. The myeloma cell line has been selected for a specific enzyme deficiency, usually hypoxanthine phosphoribosyl transferase (HPRT), to expedite the selection of spleen-myeloma hybrids. The cells are fused with polyethylene glycol (PEG) and the hybrids are selected in media containing hypoxanthine, aminopterin and thymidine (HAT). The unwanted myeloma-myeloma hybrids cannot utilize the hypoxanthine or thymidine supplied in the media and aminopterin blocks *de novo* synthesis of purines and pyrimidines. Spleen-spleen fusions cannot be maintained in culture and die off quickly. The fused hybrids, however, have the immortality of the myeloma cell and the HPRT from the spleen cell and can survive in the selection media.

Once the hybridomas are established, recloning is essential to maintain the monoclonality of the preparation. Several techniques are available and include limiting dilution, cloning in soft agar, or cloning by electronic cell sorter.[14] Screening of cell

supernatants is carried out at several stages in the whole procedure to isolate the hybridomas producing the antibody of interest. The most commonly used techniques are enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA).

Although the mouse is the most commonly used animal for hybridoma production, rat myeloma cell lines have also been established.[16] Human MAbs are more difficult to produce due to the limited number of available, non-immunoglobulin secreting human myeloma cell lines[20] and the instability of human-mouse myeloma hybrids. Other immortalization techniques have been developed to improve the production of human MAbs and include Epstein-Barr virus (EBV) transformation of sensitized human B lymphocytes, electrofusion and construction of heteromyelomas.[16] The latter two are still experimental but initial results are encouraging. The biggest obstacle in producing human MAbs is obtaining the appropriately sensitized B lymphocytes. Research in this area is also producing promising results with *in vitro* immunization of human B lymphocytes.[20]

Once the appropriate clones are isolated, stocks of the hybridoma can be deep-frozen for future use. Large scale production of milligram quantities are available by mass culturing or growth as ascites tumours in mice or rats. Mass culturing is time consuming, does not produce much MAb (1 to 10 μg/ml) and requires extensive manipulation prior to purification. Ascitic fluid is a concentrated source and produces from 1 to 5 mg/ml of MAb. Production of gram quantities of MAb requires the use of large scale, industrial fermenters where 100 μg/ml of MAb can be produced in capacities exceeding 1000 litres.[16]

Purification of the MAb from cell supernatants or ascites fluid is generally accomplished by both standard and high performance affinity chromatography. The type of affinity column chosen is usually dependent upon MAb subclass. For example, Protein A Sepharose and DEAE Cellulose can be used to purify IgG subclasses but are not effective for IgM purification.[17] Hydroxylapatite, in standard and high performance format, can purify both IgG and IgM with relative ease.[18,19] Purification is not without problems, however, as some MAbs can be inactivated by these methods and each MAb must be screened individually to chose the appropriate purification technique.

1.3 RADIOLABELS FOR IMMUNODIAGNOSIS

The development of MAb for *in vivo* use has also generated research into the choice of the most appropriate radiolabel for immunodiagnosis. Radioisotopes of iodine were first employed for labeling antibodies [9,10] but current research is aimed at labeling antibodies with the more useful radioisotopes of indium, gallium and technetium.[11]

1.3.1 RADIOIODINE

Iodine, whether stable or radioactive, is known to be highly reactive with proteins and with the availability of ^{131}I methods were soon developed to label this radioisotope of iodine into proteins.[21] The popularity of radioiodine was due to its availability in high specific activity (^{123}I and ^{131}I), its relative ease of incorporation into proteins and the accessibility of detection *in vivo* by external processes such as scintigraphy. The limiting factor for external scintigraphy, however, was the availability of an appropriate radioisotope with optimal decay characteristics and reasonable half-life (see Table 1). Iodine-125, with its low energy γ-ray (35 keV) and X-ray (Te X-rays, 27 and 31 keV) emission and long half-life (60 days), makes it unsuitable for external imaging. It has found application in *in vitro* tests such as RIA and has replaced ^{131}I for this purpose. Iodine-131 has a complex decay scheme involving several γ emissions (80 to 723 keV, 364 kev most predominant) with high energy β components and is not the ideal radioisotope for scintigraphy, either. Its 8.1 day half-life and associated β component result in a high radiation dose when injected *in vivo*. In spite of these drawbacks, ^{131}I is the most widely used of the iodine radioisotopes mainly due to its availability. Iodine-123 is by far the most useful radioisotope for imaging with its 159 keV γ-ray but its short half-life (13 hours) and limited availability inhibit its widespread use in nuclear medicine. The remaining radioisotopes of iodine do not possess adequate physical characteristics for use in radioimmunoimaging and will not be discussed here.[22]

There are a wide variety of reagents available to label radioiodine into proteins.[23] In order to make iodine reactive with proteins it must be converted to the +1 oxidation state and this is generally achieved with the use of oxidizing agents. The compound first introduced for

Table 1. Characteristics of Selected Radioisotopes Used for Radioimmunoimaging*

Radioisotope	Half-life	Decay Mode	Major γ-Ray Energy in kev (%)
^{123}I	13.2 h	E.C.[1]	159 (82.8) Te x-rays: 31 (16.0)
^{125}I**	60.1 d	E.C.[1]	35 (6.7) Te x-rays: 27 (114) 31 (26)
^{131}I	8.0 d	β^-	80 (2.6) 284 (6.1) 364 (81.2) 637 (7.3)
^{67}Ga	3.3 d	E.C.[1]	93 (38.3) 185 (20.9) 300 (16.8) 394 (4.7)
^{68}Ga	68.0 m	β^+, E.C.[1]	511 (178.2) 1077 (3.3)
^{111}In	2.8 d	E.C.[1]	172 (90.9) 247 (94.2) Cd x-rays: 23 (67.0) 26 (14.5)
$^{113}In^m$	99.5 m	I.T.[2]	392 (64.9) In x-rays: 24 (18.7) 27 (4.3)
$^{99}Tc^m$	6.0 h	I.T.[2]	140 (89) Tc x-rays: 18 (6.1)

1. E.C. - electron capture.
2. I.T. - internal transition.

* From 'Table of Isotopes 7th ed.', Lederer CM and Shirley VS, eds. John Wiley and Sons, Toronto. 1978.

** Used for experimental small animal scintigraphic studies.

labeling proteins was iodine monochloride (ICl)[13] in 1958. The main disadvantage of this technique is the incorporation of non-radioactive iodine into the protein, reducing the specific activity attainable. Chloro compounds, such as chloramine-T (Cl-T),[14] generate no-carrier-added radioactive ICl and produce high specific activity proteins. However, the strong oxidizing capacity of Cl-T and the need for reducing agents to quench the reaction can adversely affect the quality of the protein preparation. To overcome this, an insoluble chloroamide, 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Iodo-gen, Pierce),[15] was developed that produces high specific activity labeled proteins with minimal exposure to the oxidizing agent and no need for reducing agents to quench the reaction. Other techniques that have been used but are not as popular as the abovementioned reagents are free or immobilized enzyme mediated oxidation reagents using lactoperoxidase,[16] horseradish peroxidase[17] and myeloperoxidase;[18] labeling by conjugation of radioiodinated aromatic structures such as the N-hydroxysuccinimidyl ester of radioiodinated 3-(4-hydroxyphenol) proprionic acid[19] and electrolytic radioiodination.[20]

In all cases, the radioiodination of the protein involves incorporation of radioiodine into tyrosine residues and to some extent into histidine residues. The net result of this process is the electrophilic substitution of hydrogen by the iodonium ion on tyrosine and histidine molecules. This reaction is stable and irreversible at pH 7.5 to 9.0.

Although radioiodinated proteins have enjoyed wide use for many years, the main disadvantage to *in vivo* use is the active accumulation of radioiodide by the thyroid gland. High doses of $Na^{131}I$ have been used therapeutically for thyroid cancer due to the efficient extraction of iodide by this gland. The quality control of radioiodinated proteins is essential to minimize the effect of unlabeled radioiodine on the thyroid gland. Techniques have also been developed (pre-administration of KI, NaI or perchlorate) to block thyroid uptake of metabolically released radioiodine as well.

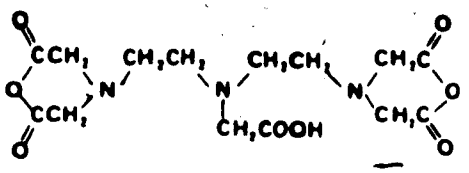
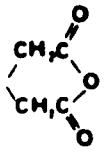
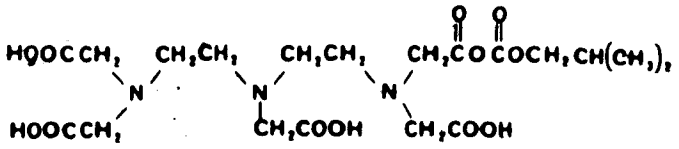
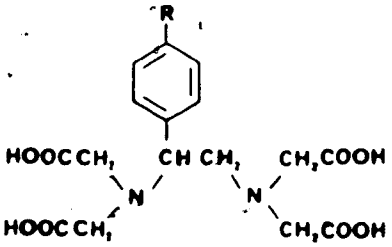
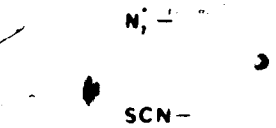
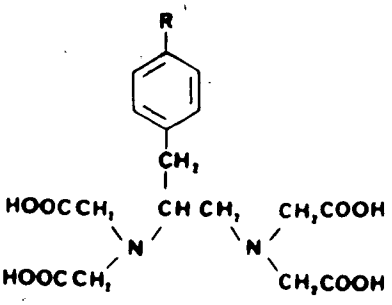
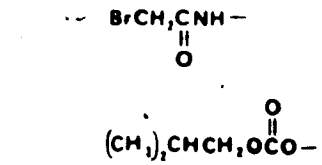
1.3.2 BIFUNCTIONAL CHELATING AGENTS

In the search for more appropriate radioisotopes to overcome the many disadvantages of radioiodine, techniques to incorporate the radioisotopes of gallium, indium and technetium have been investigated. Benisek and Richards[31] were the first to report the incorporation of a metal chelating group into hen egg white lysozyme. Subsequent research has focused mainly on the synthesis of derivatives of diethylenetriaminepentacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA) (see Table 2) although conjugation of other metal chelating agents such as transferrin,[32,33] D-penicillamine[33] and deferoxamine[34] to proteins via glutaraldehyde[32,34] or carbodiimide[33] coupling has been explored to a lesser extent.

Two derivatives of DTPA have enjoyed considerable popularity for labeling MAbs. The bicyclic anhydride, first described by Eckelman *et al.*,[36] has been adapted by Hnatowich *et al.* for labeling albumin[37] and MAbs[38] with DTPA. The mixed carboxycarbonic anhydride of DTPA was first described by Krecjareck and Tucker[39] and has been adapted by several groups[40-42] for labeling MAbs. Carboxylic anhydrides of the type described here bind to proteins through free amino groups. The delta amino group of lysine is usually the most readily avaliable for this reaction and forms a stable amide bond. Acylation of other residues, such as the imidazole group of histidine, the sulfhydryl group of cysteine and the hydroxyl group of tyrosine result in unstable bonds that are quickly hydrolyzed at neutral pH.[43] Amide bond formation is favoured by elevated reaction pH which helps to prevent these unwanted side reactions from taking place.

There are several advantages and disadvantages with the use of these two reagents as Wang *et al.*[43] pointed out in a comparative labeling study using both derivatives to conjugate ^{111}In to an anti-melanoma MAb. The *in vitro* stability testing showed that the cyclic anhydride was considerably more stable and more convenient to synthesize than the carboxycarbonic anhydride. Labeling yields, retained immunoreactivity and biodistribution in tumour bearing mice were identical for both compounds. The main advantage of the carboxycarbonic anhydride is the activation of only one carboxyl group on DTPA (under strictly controlled conditions) whereas the formation of the bicyclic anhydride ultimately involves the activation

Table 2. Currently Available Bifunctional Chelating Agents for Radiolabeling with Cationic Radioisotopes

Compound	Structure	Protein Reactive Group
1. DTPAan[1]		
-bicyclic		
-carboxycarbonic		
2. phenyl-EDTA[2]		
3. benzyl-EDTA[2]		

1. DTPAan - diethylenetriaminepentaacetic acid anhydride
2. EDTA - ethylenediaminetetraacetic acid

of two carboxyl groups with an increased chance for crosslinking and polymerization of protein molecules. Crosslinking can be a problem with the carboxycarbonic anhydride if synthetic reaction conditions are not strictly controlled to produce activation of only one carboxyl group on DTPA.[44] Other methods to incorporate DTPA into proteins include the use of direct carbodiimide coupling to MAbs[46] and the preparation of the N-hydroxysuccinimide ester of DTPA[47,48] both of which have met with limited success.

The derivatization of EDTA was first described by Goodwin *et al.*[49] in 1973 and was used to label albumin and fibrinogen.[50,51] The original modification of EDTA involved the introduction of a nitrophenyl group onto one of the carbons of the EDTA backbone and subsequent derivatization of the nitro group to a diazonium salt. The diazo functionality could then react with terminal amino groups on proteins providing a method of introducing radioactive metal ions into the protein. Meares *et al.*[52] and Yeh *et al.*[53] demonstrated the superior *in vitro* and *in vivo* stability of proteins labeled with ^{111}In via derivatized EDTA as compared to derivatized DTPA. The major drawbacks to the use of the diazo-phenyl-EDTA were the complicated synthetic scheme and the fact that proteins labeled with the diazo compound were cleared faster than equivalent radioiodinated proteins.[54] The aromatic nitro group can be modified by a number of methods other than diazotization, such as acylation and alkylation,[55] to produce the reactive side chain. A new synthetic scheme was devised by Yeh *et al.*[56] that allowed the production of nitro-phenyl- and nitro-benzyl-EDTA using amino acid precursors by a much simpler route than that first described by Sundberg *et al.*.[51] As well, the use of an alkylating reagent on the reactive side chain produced a homogeneously labeled product that exhibited plasma clearance that was identical to equivalent radioiodinated proteins.[57] The p-bromoacetamido- and isothiocyanate-derivatives of nitro-benzyl-EDTA were adapted by Meares *et al.*[51] and Goodwin *et al.*[60] for the labeling of MAbs. The haloacetamide reagent was preferred although MAbs contain few free sulfhydryl groups and it was speculated that reaction with free amino groups was the dominant site of attachment. The derivatized EDTA compounds have also been used to label bleomycin with ^{111}In and used in clinical trials for diagnosis of head, neck and lung cancers.[59] Other bifunctional chelating

agents have been described, for example, N'-(p-diazoniumbenzyl)-N,N,N'',N'''-diethylene-triaminetetraacetic acid (DTTA, a derivative of DTPA)[61] and cryptate complexes such a 6(p-bromoacetamidobenzyl)-1,4,8,11-tetraazacyclotetradecane-N,N',N'',N'''-tetraacetic acid as a bifunctional chelate to label copper to proteins.[62]

The elements most commonly applied to the labeling of MAbs and other proteins with bifunctional chelating agents are radioisotopes of indium, gallium and technetium (see Table 1).

By far the most widely used radioisotope is ^{111}In. It possesses a 2.8 day half-life and suitable γ emissions (172 and 247 kev) for imaging with chelate labeled MAbs. It is available in high specific activity as [illegible] and is most commonly produced by a (p,n) reaction on ^{111}Cd in cyclotrons. $^{113}In^{m}$ [illegible] lived radioisotope (1.7 hours) available from a generator source ($^{113}Sn \rightarrow {}^{113}In^{m}$, $t_{\frac{1}{2}}$ = [illegible] hrs) that has not been widely applied to chelate labeled MAb studies but has been used as the EDTA and DTPA chelates for brain imaging and renal function studies.[63]

The radioisotopes of gallium that hold interest for chelate labeled MAbs are ^{67}Ga and ^{68}Ga. ^{67}Ga has a 3.3 day half-life and suitable γ emissions for external scintigraphy (93, 185, 300 and 394 kev). It has not been as widely used for chelate labeled MAbs as ^{111}In but can be produced in high specific activity from cyclotron sources by a (p,n) reaction on ^{67}Zn.[64] ^{68}Ga is a short-lived, generator produced, positron emitting radioisotope of gallium that has been used in tomographic studies of liver and brain.[63] ^{68}Ga has been coupled to albumin via the carboxycarbonic anhydride of DTPA[64,65] and used to measure cerebral blood pool by positron tomography.

The short-lived radioisotopes of gallium and indium have limited usefulness for scintigraphic cancer diagnosis with chelate labeled MAbs. However, both ^{111}In and ^{67}Ga possess excellent characteristics for application to diagnosis with their intermediate half-lives and suitable γ emissions.

The third radioisotope employed for chelate labeled MAbs is $^{99}Tc^{m}$. This is a generator produced ($^{99}Mo \rightarrow {}^{99}Tc^{m}$, $t_{\frac{1}{2}}$ = 67 hours), short-lived (6 hours) radioisotope with a highly

abundant γ ray at 140 kev that is ideal for external scintigraphy. It is widely employed as various complexes in nuclear medicine for blood pool imaging, brain scanning, liver, spleen, bone and bone marrow imaging.[46] The major drawbacks for the use of $^{99}Tc^{m}$ for labeling MAbs relate to its half-life and the complicated carrier-free chemistry of the radioisotope. With intact MAbs, the blood clearance is such that optimum imaging times range from 3 to 5 days post injection and this corresponds to a time frame when $^{99}Tc^{m}$ would have long disappeared due to physical decay. More promising results might be obtained with MAb fragments, where faster blood clearance allows tumour visualization at 24 hours, but again physical decay limits the extent of imaging. The chemistry of $^{99}Tc^{m}$ is such that reduction of the anionic pertechnetate species is required to complex it to proteins, either directly[47] or through chelating agents.[48] Although the direct method is simple and easy to perform, the reduction is usually achieved by the addition of stannous chloride and unless precautions are observed the resulting $^{99}Tc^{m}$-MAb complex is unstable and possesses reduced immunoreactivity. To label $^{99}Tc^{m}$ to chelate labeled MAbs also requires the presence of a reducing agent and although this method should produce only chelated $^{99}Tc^{m}$, Paik *et al.*[49] have demonstrated that DTPA labeled MAbs coupled with $^{99}Tc^{m}$ in the absence of free DTPA, contain both direct-labeled and chelated radioisotope. The direct labeled $^{99}Tc^{m}$ proved to be unstable and was easily removed.

Further research is ongoing to provide a stable $^{99}Tc^{m}$ labeled MAb for tumour imaging but the chemistry involved has so far precluded its widespread use in this area and restricted the choice to radioisotopes of gallium and indium.

1.4 *IN VIVO* APPLICATIONS OF RADIOLABELED MAbs

The use of radiolabeled MAbs for tumour diagnosis has rapidly expanded in the last five years, due mainly to the development of hybridoma technology (as previously described) and the use of MAbs for identifying tumour-associated antigens. Animal models, including human tumour xenografts in nude mice, have produced much of the data on the distribution of radioiodine- and radioindium-labeled MAbs and give insight into the expected results when these radiolabeled MAbs are used *in vivo* in human studies. The identification of tumour associated antigens has led to the investigation of a number of different cancer types. Table 3 summarizes the data for animal model systems for the past five years. Many of the MAbs investigated in these animal models have been applied to human clinical trials based upon the results obtained in these studies. By far the two cancers that have been the focus of research in animal models are the colorectal carcinomas and the melanomas with human tumour xenografts in nude mice. To date, 18 MAbs have been generated that localize in colorectal carcinomas in animals and 10 of these are directed against purified carcinoembryonic antigen (CEA). The remainder have been generated against a variety of human colon cancer cells and two MAbs, 791T/36 and C/24/1/39/11/1 generated against an osteogenic sarcoma, show excellent binding to human colorectal cells. The majority of MAbs produced to CEA or human colorectal cancer cells (HCC cells) are of the IgG_1 subclass. In a few cases, comparative studies between radioindium- and radioiodine-labeled MAbs have been made as well as comparisons between intact IgG, $F(ab')_2$ and Fab fragments of the IgG. The general trend indicates that radioindium-labeled MAbs are accumulated by the tumour to a greater extent than radioiodine-labeled MAbs, producing a higher tumour : background ratio and better quality images.[31,69-71,77,81,82] As well, background subtraction was not required for radioindium as it was cleared more rapidly from the blood than radioiodine.[69,71] Liver uptake was a recurrent problem with radioindium labeled MAbs and possibly reflects the different metabolic fates of radioindium and radioiodine. Liver uptake did occur with one of the radioiodinated MAbs[83] and this was attributed to immune complex formation with circulating antigen. Overall, $F(ab')_2$ fragments were preferred to intact IgG[31,69,71,73,84] due to their rapid

Table 3. Radioimmunodetection of Cancers in Animals using Polyclonal and Monoclonal Antibodies.

MAb (Isotype)	Antigen	Production Species	Radiolabel (Method)	Immunoassay Method	Result	Cancer Type (Host)	Results and Comments	Reference
202 (IgG-1) 23 (IgG-1) 192 (IgG-1) 35 (IgG-1)	purified CEA[1] purified CEA[1] purified CEA[1] purified CEA[1]	mouse	^{131}I (ICl, Iodo-bead) ^{111}In (DTPAan) ^{90}Y (DTPAan)	CBA[2] CEA-AC[2]	21-30% 50-78%	HCC[4] -Col12 (nude mice)	-F(ab')$_2$ compared to intact IgG -F(ab')$_2$ higher in tumour -F(ab')$_2$ best at 2 - 3 d -Intact IgG best at 4 - 5 d -^{111}In better than ^{131}I	69 - 72
CEJ-30.6 (IgG-1	purified CEA[1]	mouse	^{125}I (lactoperoxidase) ^{111}In (DTPAan) ^{75}Se (metabolic)	CBA[2]	70%	HCC[4] -T380 -T379 -T157 -T84 (nude mice)	-Increasing tumour size, decreases uptake of ^{125}I and ^{111}In -^{111}In uptake in liver -^{125}I rapidly excreted	73 - 76
αCEA[1] (IgG-1)	purified CEA[1]	mouse	^{125}I, ^{131}I (lactoperoxidase, Cl-T) ^{111}In (DTPAan)	CBA[2] CEA-AC[2] -I-MAb -In-MAb(1:1) -In-MAb(20:1)	59% 70% 92% 79%	HCC[4] -GW39 (syrian golden hamster HCC[4] -HT29 (nude mice)	-F(ab')$_2$, Fab compared to intact IgG -F(ab')$_2$ best at 2 d -^{111}In better than ^{125}I, ^{131}I -tumours visible 24 h	38, 77
T84 (IgG 1) 66A3 (IgG-1) 1H11 (IgG-1)	purified CEA[1] purified CEA[1] purified CEA[1]	mouse	^{111}In (DTPAnhsuc-ester)	ELISA[1] (CEA[1]) rxn 36,72:1 rxn 180:1	100% 36%	HCC[4] -LS174T -WiDr -SW403 (nude mice)	-increasing liver uptake with increasing CEA secreted by tumour -best image 72 h	78, 79
AUA1 (IgG-1)	HCC[4] LoVo	mouse	^{125}I, ^{131}I (Cl-T, Iodo-gen)	?	?	HCC[4] -HT29 (nude mice)	-compared location of tumour: IM > IP > SC -best image at 48 h in IM tumour	80

Table 3. ...continued

791T/36 (IgG-2b)	HOS[a] -791T	mouse	^{125}I, ^{131}I (Iodo-gen)	CBA[b] -^{131}I-MAb	30%	HCC[c] -LS174T	-subt required for ^{131}I -^{111}In better than ^{131}I	81, 82
C/24/1/- 39/11/1 (IgG-1)	HCC[c]	mouse	^{111}In (DTPAan)	-^{111}In-MAb	34-49%	-HT29 -HCT-8 (immunosup. mice)	-best image at 3 to 6 d	
17-1A (IgG-2a)	HCC[c]	mouse	^{125}I, ^{131}I (Cl-T, Iodo-gen)	CBA[b] -Iodo-gen	13%	HCC[c] -HRT18	-no subt -tumour uptake to 9 d	83
19-9 (IgG-1)	HCC[c] (CA19-9)	mouse		-Cl-T	9%	-HT29 (nude mice)	F(ab')$_2$ better at early times -19-9 liver and spleen activity due to circ. immune complexex	
C2032 (IgG-2a)	HCC[c]	mouse	^{125}I, ^{131}I (Iodo-gen)	CBA[b]	35-40%	HCC[c] -SW948 -SW1122 (immunosup. mice)	-F(ab')$_2$ compared to intact IgG -best image at 4 to 5 d -no subt required for F(ab')$_2$	84
38S1 (IgG-1)	purified CEA[b]	mouse	^{131}I (Cl-T)	?	?	HCC[c] -LS174T -HT29 (nude mice)	-best uptake 4 to 5 d -+ve corr. to CEA content and uptake -no subt required -no corr. to tumour size	85
W632 (IgG-2?)	HTC[b] membrane	mouse	^{3}H (metabolic)	CBA[b]	qualitative only	HCC[c] -HT29 (nude mice)	-distribution study -high uptake in liver and kidney due to circ. immune complexes	86
8.2 (IgG-1)	HM[d] -p97	mouse	^{125}I, ^{131}I (Cl-T)	CBA[b]	56-83%	HM[d] -2169 (nude mice)	-Fab compared to intact IgG -increased Fab dose increased tumour uptake -Fab better than intact IgG	87, 88
96.5 (IgG-2a)	HM[d] -p97	mouse						
48.7 (IgG-1)	HM[d] -HMWA[d]	mouse	^{99m}Tc (SnCl$_2$ redn.)	Affinity Chrom.	60-80%	normal mice	-compared direct and indirect labeling -indirect comparable to ^{111}In-MAb	68

Table 3. ...continued

9.2.276 (IgG-1)	HM[8] (250 kd gp)	mouse	^{111}In (DTPAan)	RIA[10] (cell extract)	> 80%	HM[8] -FMX-Met (nude mice)	-tumour peaks at day 4 -high liver and tumour uptake -best image after 4 d	89, 90
225.28S (IgG-2a)	HM[8] -HMWA[9]	mouse	^{111}In (DTPAan)	CBA[2] ELISA[1] < label. > label.	27% titer: 1:1024 1:128	HM[8] -LG-2 (nude mice)	-high liver, kidney, tumour uptake -best image at 4 d -liver activity may be due to circ. immune complexes	91
αGD2 (IgM)	B lympho-cytes from mel. pt.	human (EBV trans.)	^{131}I (Cl-T)	CBA[2] IA[11] < label. > label.	58% titer: 1/2000 1/200	HM[8] -SOM14 -SOM24 (nude mice)	-high liver, spleen, kidney uptake -tumour visible at day 4	92
αH Mel (poly IgG)	HM[8] -JH-1	monkey	^{123}I, ^{131}I (Cl-T)	?	qualitative only -suggests problems	HM[8] (nude mice)	-high background -best images 6 to 7 d -MAb not better than poly IgG	93
αH Mel (poly IgG)	HM[8] -JH-1	human						
Nu4B (IgG(?))	HM[8] -JH-1	mouse						
791T/36 (IgG-2b)	HOS[6] -791T	mouse	^{111}In (DTPAan)	CBA[2]	34-49%	HOS[6] -788T -791T	-no subt required -tumour visible at 24 h -best image at 3 to 6 d	94, 95
LICR-LON/HT13	HMT[12] -HX39/7	mouse	^{123}I, ^{131}I (Cl-T)	CBA[2] Gel Chrom.	qualitative only no aggreg.	HMT[12] -HX39/7 -HX112/3 -HX57/17 (immunosup. mice)	-no subt required -rapid blood clearance -specific tumour uptake -tumour index highest at 48 h -uptake only in viable tumour	96 - 98
BA-1 (IgM)	HLL[13]	mouse	^{131}I (ICl) ^{131}I-MAb ^{111}In (DTPAan) ^{111}In-MAb	CBA[2]	35% 30%	normal mice	-high liver, spleen and kidney uptake for ^{111}In -prelim. data in normal mice	69

Table 3. ...continued

B6.2 (IgG-1)	HBC[14] -membrane	mouse (Iodo-gen)	^{125}I-	CBA[3]	qualitative only	Clauser human breast carcinoma (nude mice)	-increased clearance of B6.2 compared to nonsp. IgG-1 -good tumour uptake	99
10-3D2 (IgG-1)	HBC[14] -BT20	mouse	^{111}In (DTPAan)	?	?	HBC[14] -BT20 -HS758T (nude mice)	-F(ab')$_2$ better than intact IgG for imaging -tumours visible at 24 h	40
αHBsAg[13] (?)	HBsAg[13]	mouse	^{125}I (Cl-T)	RIA[10] (HBsAg[13])	70%	PLC[16] -PRF/5 (nude mice)	-small localization index -would not distinguish cancer from infections or circ. HBsAg[13]	100
19-24 (IgG-1)	MFH[17]	mouse	^{125}I (Cl-T)	ELISA[3] CBA[3]	-equivalent to unlab. MAb 90%	HFS[18] -HT-1080 (nude mice)	-best image at 6 d -clearance half-life 1.6 d	101, 102
HMFG-1 (IgG-1) HMFG-2 (IgG-1)	delipida HMFC membr		I, ^{125}I gen, Cl-T)	RIA[10] ELISA[3]	qualitative only qualitative only	ovarian xenografts	-maximum uptake at 6 d	103
103A (IgG-1)	Raus murine leukemia virus		I (Cl-T) ^{111}In (DTPAan)	?	?	Rauscher erythro-leukemia (Balb/c mice)	-fragment compared to intact IgG -fragments did not improve image -maximal uptake at 6 h	104 - 106
14AC1 (IgG-2a)	rat glioma -79FRG-41	mouse	^{125}I (Cl-T)	?	?	rat glioma -79FRG41 (nude mice)	-tumour visible at 24 h -rapid deiodination *in vivo*	107
D3 (IgG-1)	L10 hepato-carcinoma	mouse	^{125}I (Bolton-Hunter) ^{111}In (DTPAan)	CBA[3] ^{125}I-MAb ^{111}In-MAb	51-67% 63-67%	L10 hepato-carcinoma (guinea pig)	-^{125}I-MAb cleared faster than ^{111}In-MAb -high liver, spleen, kidney tumour uptake of ^{111}In-MAb	41, 89, 108

Table 3. ...continued

MIA (IgG-1)	MI[20] myo-sarcoma	mouse (SnCl, red)	$^{99}Tc^{m}$ (MI[20] ext)	ELISA[5]	qualitative only	MI[20]-myo-sarco (DBA/2 mice)	-high background -some tumour uptake -limited to 24 h scan	109
HDP-1 (?)	DNP[21]	mouse	^{111}In (DTPAan)	AC[3]	85%	DNP-agarose loc. in lung (rat)	-claims saturable mechanism for ^{111}In-MAb accumulation in liver	110
αIA-k (IgG-2a)	Balb/k B lympho-cytes	mouse	^{111}In (BrAcB-EDTA) ^{131}I (Cl-T)	CBA[2]	77-89%	normal mice -lymph nodes of IA-k	-^{131}I rapidly excreted -best image by s.c. injection in foot pad	58, 60

List of Abbreviations

1. CEA - carcinoembryonic antigen
2. CBA - cell binding assay
3. AC - affinity chromatography
4. HCC - human colorectal carcinoma
5. ELISA - enzyme linked immunosorbent assay
6. HOS - human osteogenic sarcoma
7. HTC - human tonsil cell
8. HM - human melanoma
9. HMWA - high molecular weight antigen
10. RIA - radioimmunoassay
11. IA - immune adsorbance
12. HMT - human mammary tumour
13. HLL - human leukemias & lymphomas
14. HBC - human breast carcinoma
15. HBsAg - hepatitis B surface antigen
16. PLC - primary liver cancer
17. MFH - malignant fibrous histiocytoma
18. HFS - human fibrosarcoma
19. HMFG - human milk fat globule
20. MI - methylcholanthrene induced
21. DNP - dinitrophenol

blood clearance and early delineation of the tumour compared to intact IgG.

The melanoma antibodies that have been studied involve the distribution of various polyclonal and monoclonal preparations in human melanoma xenografts in nude mice. Several well defined melanoma associated antigens have been described, p97 - a 97000 kd membrane antigen[87] and a membrane, high molecular weight antigen (HMWA).[87,89,91] The comparison between radioindium- and radioiodine-labeled MAbs has not been as extensive as with the colorectal MAbs but again high liver uptake was observed when ^{111}In was used to label these proteins[89,91] and is attributed to sequestration of circulating immune complexes. Fragment IgG studies were carried out in one case and Fab was superior to intact IgG especially when the dose was serially increased.[87] One study involved the use of a human anti-melanoma MAb produced by EBV transformation of B lymphoblastoid cells sensitized to GD_2[92] and showed good tumour localization after 4 days. Two polyclonal IgG anti-sera, produced in monkey and human, were compared to an anti-melanoma MAb[93] and in this particular case, the MAb showed no better localization than the polyclonal IgG preparations.

The remainder of the animal studies employ one or two MAbs in various tumour systems (both human tumour xenografts and specific animal models). Again, the overall comparison of radioindium- and radioiodine-labeled MAbs favours tumour accumulation of ^{111}In.[41,89,94,95,104,106,108] ^{111}In-labeled MAbs required no background subtraction compared to radioiodine-labeled MAbs although studies with radioiodinated MAb LICR-LON/HT13[94,95] indicated that no subtraction technique was required to visualize the tumour. In those studies that included IgG fragments,[40,104-106] one indicated that F(ab')$_2$ improved the imaging results[40] and the others did not.[104,106] MAb 791T/36, the anti-human osteogenic sarcoma (HOS) antibody, found application in both colorectal and osteogenic sarcoma xenografts in nude mice.[81,82,94,95] In experiments using MAb B6.2, an anti-human mammary tumour (HMT) antibody,[99] increased clearance of the specific radioiodinated MAb was observed compared to non-specific MAb. This was attributed to specific metabolism of the radioiodinated MAb B6.2 by the tumour cells with release of radioiodine. In spite of the accelerated clearance, good imaging results were obtained.

Many of the MAbs described for the animal studies have been applied in human clinical trials for the diagnosis and treatment of cancer. Table 4 summarizes data from the last five years available for MAbs tested in humans. With most of the clinical trials, preliminary data acquired in animals prompted the application of these MAbs to human trials.

The antibodies investigated in human trials are divided into six cancer types with colorectal, gynecological and melanoma tumours ranking as the top three. For colorectal carcinomas, 12 polyclonal and monoclonal preparations labeled with both radioindium and radioiodine have been studied for their ability to localize primary and metastatic cancer although radioiodine predominates as the imaging label. Most MAbs have been generated against whole human colorectal carcinoma (HCC) cells[113-120] but several polyclonal preparations against CEA[123-126] and one MAb to CEA[121] have also been studied. The MAbs are predominantly of mouse origin and of a variety of subclasses with the exceptions being a single rat MAb and the polyclonal preparations. Comparisons between radioindium- and radioiodine-labeled preparations are few since most human studies were conducted using ^{131}I. Two studies with MAb 791T/36[111,112] suggest that ^{111}In generated superior images with no subtraction as compared to ^{131}I. Liver uptake of ^{111}In-labeled MAbs is also apparent in the human studies and this uptake obscures metastatic disease present in this organ. No comparison had been made between the polyclonal and monoclonal preparations to determine if the MAbs were superior for image quality and success of detection of the disease. The use of fragments for colorectal imaging is not as extensively documented as for the animal studies but experiments with MAbs 19.9 and 17.1A[115-119] have shown the superiority of $F(ab')_2$ fragments for imaging colorectal carcinoma, especially when they are labeled with ^{111}In.

For breast carcinoma, ovarian and cervical carcinoma as well as testicular carcinoma, 7 MAbs raised against various tumour associated antigens have been used. All are of mouse origin and whole human breast carcinoma (HBC) cells as well as membrane extracts have been used as antigens. MAb 791T/36, the anti-HOS MAb described previously,[111,112] reacts not only with colorectal carcinoma but also with primary and metastatic breast carcinoma[129,130] as well as a variety of bone malignancies.[111] Comparison between

Table 4. Radioimmunodetection of Cancers in Humans using Polyclonal and Monoclonal Antibodies.

MAb (Isotype)	Antigen	Production Species	Radiolabel (Method)	Immunoassay Method	Immunoassay Result	Cancer Type	Results and Comments	Reference
791T/36 (IgG-2a)	HOS[1] -791T	mouse	^{131}I (Iodo-gen) ^{111}In (DTPAan)	CBA[2] Immune precipitation	34-40% 90-96% precipitated with αM IgG	colorectal carcinoma	-used background subt -^{111}In superior to ^{131}I with high uptake in liver -better images at 72 h (48h)	111,112
250-30.6 (IgGG-2b	HCC[3] -HT-29	mouse	^{131}I (Cl-T)	?	qualitative only no reduction reported	colon adeno-carcinoma	-90% success rate -imaging at 48 h	113, 114
17-1A (IgG-2a)	HCC[3]	mouse	^{131}I (Iodo-gen)	CBA[2]	7-50%	colorectal carcinoma	-used background subt -69% imaged -histochemistry correlated with scan -best images for MAb at 6-7 d, for F(ab')$_2$ at 4-5 d	115-118
19.9 (IgG-1)	HCC[3] -CA 19.9 (ganglioside)	mouse	^{131}I (Iodo-Gen) ^{111}In (DTPAan)	competition assay	100%	colorectal and pancreatic carcinoma	-used background subt -10/13 imaged with ^{131}I, 8/12 for ^{111}In -best images at 48 h (24,72 h)	115, 119
YPC 2/12.1 (IgG-2a)	HCC[3] -membrane preparation	rat	^{131}I (Cl-T)	CBA[2] HT-29 cells	qualitative only no reduction reported	colorectal and others	-13/16 imaged for colorectal, overall 16/27 imaged -imaged at 24 and 48 h	120
VII-23e (IgG-1) VII-37a (IgG-2?)	purified CEA[4]	mouse	^{131}I (?)	RIA (CEA[4])	?	colorectal and medullary thyroid carcinoma	-used background subtraction -imaged at 5 h to 48 h	121

Table 4. ...continued

AUA1 (IgG-1)	PLAP[4]	mouse	^{131}I, ^{111}I (Iodo-gen)	ELISA[1] RIA[1]	100% 100%	colorectal, ovarian and breast carcinoma	-no imaging -demonstrated AUA1 on tumor sections by immunohistochemical staining and radiolabeled MAb binding	122
αCEA[4] (poly IgG)	purified CEA[4]	goat	^{131}I (Cl-T)	CEA-AC[4]	70%	colorectal carcinoma	-used background subtraction -overall 56/65 imaged -imaged at 24 and 48 h	123, 124
αCEA[4] (poly IgG)	purified CEA[4]	sheep	^{131}I (ICI) ^{111}In (DTPAan)	RIA[1] (CEA[4])	qualitative only no reduction reported	colorectal and others	-overall 28/31 imaged -^{131}I equivalent to ^{111}In -imaged at 1 to 9 d -noteworthy uptake of ^{131}I in bladder and ^{111}In in liver	125
PK1G (poly IgG)	purified CEA[4]	goat	^{131}I (Cl-T)	?	?	colorectal carcinoma	-lyposomally entrapped second antibody (LESA) -overall 3/5 imaged -imaging at 2, 4, 24 and 48 h	126
NDOG$_2$ (IgG-2b)	PLAP[4]	mouse	^{131}I (Cl-T)	ELISA[1] (on PLAP[1])	90-95%	ovarian carcinoma	-best images post micturition -subt produced artefacts -correlates to CT, etc. -imaged at 4m, 4 & 20 h	127
H17E2 (IgG-1)	PLAP[4]	mouse	^{111}In (DTPAan)	?	qualitative only	ovarian, cervical, testicular	-no subt required -high liver uptake -PLAP +ve tumours produced no false +ve or -ve -better than ultrasound, CT -imaged after infusion, 1 to 7d	128
791T/36 (IgG-2b)	HOS[1] -791T	mouse	^{131}I (Iodo-gen) ^{111}In (DTPAan)	CBA[1] -^{131}I-MAb -^{111}In-MAb	44% 35-50%	1° & 2° breast carcinoma, various gynecological	-subt required for ^{131}I -^{111}In-MAb superior -liver uptake of ^{111}In -imaged at 48 to 72h	129, 130
3E1.2 (IgM)	HBC[4]	mouse	^{131}I (Cl-T)	?	qualitative only	breast carcinoma	-administered s.c. -+ve on affected lymph nodes -imaged at 16 to 24 h	113

Table 4. ...continued

HMFG-2 (IgG-1)	delipidated HMFG[10] membrane	mouse	^{123}I, ^{125}I, ^{131}I (Iodo-gen)	ELISA[1] -T47D	70 to 95%	ovarian	-tumour image best at 8-12 h -no subt, indwelling catheter -high excretion -imaged at infusion, 4,8 & 24 h	103, 131
AUA1 (IgG-1)	PLAP[9]	mouse	^{125}I, ^{131}I (Iodo-gen)	ELISA[1] RIA[1]	qualitative only 100%	breast, ovarian, colorectal carcinomas	-no images -+ve by immunoperoxidase staining -suggest late imaging	122
LICR-LON-M8 (IgG-1)	delipidated HMFG[10] membrane	mouse	^{123}I, ^{125}I, ^{131}I (Iodo-gen) ^{111}In (DTPAan)	CBA[1] -MCF7	qualitative only	1° & 2° breast carcinoma	-no subt required -I-MAb did not detect bone or soft tissue mets -In-MAb detected bone but not soft tissue mets -noteworthy uptake of ^{111}In in liver	132
225.28S (IgG-2a)	HM[11] -HMWA[11]	mouse	^{123}I (Iodo-gen) ^{99m}Tc (direct) ^{111}In (DTPAan)	CBA[1]	80-100%	malignant melanoma stage III-IV	-subt required for ^{123}I -F(ab')$_2$ superior to intact IgG -imaged at 30 m, every 2 h to 8h, 24 & 48 h	133, 134
p96.5 (IgG-2a)	HM[11] p97	mouse	^{123}I (Iodo-gen) ^{111}In (DTPAan)	CBA[1] ^{123}I-MAb ^{111}In-MAb	qualitative only 35-50%	malignant melanoma	-subt required for ^{123}I -Fab superior to intact IgG -increased Fab dose produced better images -^{111}In uptake in liver	135-139
48.7 (IgG-1)	HM[11] -HMWA[11]	mouse (Cl-T)	^{131}I	CBA[1]	qualitative only	metastatic malignant melanoma	-compared to Fab p96.5 -increased blood clearance -required subt -imaged at 48 h	139, 140
aH-HCG[11] (poly-IgG)	HCG[11]	goat	^{131}I (Cl-T)	RIA[1] (HCG or AFP)	qualitative only	testicular carcinoma	-required subt -imaged at 24 & 48 h -imaged sites not seen with CT, ultrasound	141
aH-AFP[11] (poly-IgG)	cord blood AFP[11]	goat						

Table 4. ...continued

791T/36 (IgG-2b)	HOS[1] -179T	mouse	^{131}I (Iodo-gen) ^{111}In (DTPAan)	CBA[2]	44%	various bone malignancies	-required subt for ^{131}I -^{111}In comparable to ^{131}I -did not see soft tissue mets -imaged at 48 to 72 h	142
LGL-1D6 (?)	HG[14]	human	^{131}I (Cl-T)	?	?	frontal lobe glioma	-MAb from B lymphocytes of glioma patient -1st use of human α-glioma MAb -successful imaging -imaged at 24 & 48 h	143

List of Abbreviations

1. HOS - human oseteogenic sarcoma
2. CBA - cell binding assay
3. HCC - human colorectal carcinoma
4. CEA - carcionembryonic antigen
5. RIA - radioimmunoassay
6. PLAP - placental alkaline phosphatase
7. ELISA - enzyme linked immunosorbant assay
8. AC - affinity chromatography
9. HBC - human breast carcinoma
10. HMFG - human milk fat globule
11. HM - human melanoma
12. HMWA - high molecular weight antigen
13. AFP, HCG - alphofetoprotein, human chorionic gonadotropin
14. HG - human glioma

radioindium- and radioiodine-labeled MAbs has been investigated more thoroughly with this group of MAbs[129,130,132] compared to the colorectal MAbs and again, ^{111}In is favoured over radioiodine because there is no need for subtraction techniques and the tumour : background ratio for ^{111}In was higher than radioiodine.[128,130,132] MAb-3E1.2, an IgM, was injected subcutaneously to facilitate imaging of affected lymph nodes in primary breast carcinoma.[131] When ^{123}I was used as the radioiodine label for MAb NDOG$_2$, the best images for ovarian carcinoma were obtained after micturition[127] and other studies using ^{123}I-labeled MAb HFMG2 eliminated bladder activity by the use of an indwelling catheter.[101,133] In both cases, subtraction was not performed and successful localization of metastatic spread was achieved. Imaging time was limited to 24 hours due to physical decay of the radioisotope and well short of the recommended imaging times of 4 to 5 days for ^{131}I- or ^{111}In-labeled F(ab')$_2$ fragments and 6 to 7 days for intact IgG labeled with these radioisotopes. Liver uptake for ^{111}In was also noted with these MAbs and varying explanations for increased liver activity have been put forth and range from the formation and sequestration of circulating immune complexes, binding of the MAbs through their carbohydrate moieties to liver lectins or simply, transchelation of ^{111}In to transferrin.[132] The latter explanation can apply only to ^{111}In but the circulating immune complexes and liver lectin binding are also applicable to radioiodine-labeled MAbs. The liver is a major site of protein catabolism and dehalogenation takes place very rapidly in this organ.[66,73] Released radioiodine from the MAb would be cleared from the liver and this is consistent with the findings of the studies cited here. ^{111}In with its chelate label is more stable to degradation by the liver and remains attached to the MAb for longer periods of time compared to radioiodine. So, although the radioiodinated MAbs may also be accumulating in the liver, rapid dehalogenation and release of the radioiodine from the liver reduces the background observed in this organ compared to that for ^{111}In.

The melanoma antigens have been extensively studied by Larson *et al.*[7,14] and Buraggi *et al.*[133,134] using only three mouse MAbs. Comparison of radioindium- and radioiodine-labeled MAbs has not been extensively investigated and in two cases, ^{111}In proved

superior[133,134] whereas in another it was no better than radioiodine.[135] The melanoma MAbs are a good example of the superiority of fragments of IgG, especially Fab. All studies preferred the use of Fab and Larson *et al.*[139] have successfully applied Fab 96.5 for targeting large doses (3700 MBq or more) of ^{131}I to melanoma *in vivo*. All radioiodinated MAbs required background subtraction and most imaging was obtained at 24 to 48 hours. Increased sensitivity was reported with ^{111}In-labeled Fab 96.5[135] where lesions of 1 to 2 cm in diameter could be detected compared to 2 to 4 cm for ^{131}I-labeled Fab 96.5[139] and 1.5 cm or greater for Fab 48.7.[139] At the present time, both Fab and F(ab')$_2$ fragments of melanoma MAbs are routinely used in human clinical trials.

The remaining MAbs and polyclonal preparations are used for imaging of testicular carcinoma, various bone malignancies and brain tumours. Two polyclonal IgG preparations have been used for imaging testicular cancer,[141] and were produced in goats using either human chorionic gonadotropin (HCG) or human cord blood alphafetoprotein (AFP). The choice of which preparation to use was based on whether or not elevated levels of HCG or AFP could be detected in the patient's serum. Although only 3/5 patients were imaged as positive with the anti-sera, the polyclonal IgG revealed sites undetected by other techniques. MAb 791T/36 was used in assessing various bone malignancies[142] and comparison of ^{111}In-labeled MAb 791T/36 with ^{131}I-labeled MAb 791T/36 produced equivalent results and neither preparation was able to localize soft tissue metastatic disease. For imaging brain tumours, the use of MAbs has been severely limited due to difficulties in defining brain tumour-associated antigens and the fact that most malignant brain tumours (especially gliomas) are extremely heterogeneous.[144] Many MAb preparations are used today primarily for immunohistochemical studies of malignant brain tumours to aid in defining brain tumour-associated antigens. However, one MAb, produced by EBV transformation of B lymphocytes found in a patient's glioma, were used to successfully localize recurrent disease in the same patient using ^{131}I, thus demonstrating the feasibility of using radiolabeled MAbs for localizing brain tumours.[143]

The use of radiolabeled MAbs for tumour diagnosis presents several challenges to investigators in this field today. These are related to quality control of the radiolabeled MAb preparation particularly with respect to the effect of the radiolabel on the immunoreactivity of the MAb. ^{131}I as a radiolabel can induce reduced immunoreactivity not only through the method of attachment but also can inflict radiation damage due to the β component of its decay. Strict control of the quality of radiolabeled MAb used *in vivo* ensures the accumulation of accurate data. Radiolabeling techniques must be standardized for each MAb as the sensitivity of the MAb to the techniques used may vary from one to the next.

The dose of MAb is also of important consideration. Larson *et al.*[11] found that increasing the amount of Fab administered improved the image quality obtained and they routinely use doses in excess of 20 mg for radioimmunoimaging. The induction of human anti-mouse antibodies (HAMA) becomes significant with the use of large MAb doses. In some cases, sensitivity responses in patients who had received large amounts of MAb necessitated the cessation of imaging studies. This particular point supports the need for human MAbs which could reduce the risk of adverse reactions to these preparations.

The future of radioimmunoimaging in nuclear medicine will depend upon the accurate identification of tumour associated antigens, the use of radiolabels that do not affect the integrity of the MAb and the development of non-immunogenic MAb preparations. These parameters must all be addressed if this technique is to become a routine tool for cancer diagnosis.

2. MATERIALS AND METHODS

2.1 PREPARATION OF BIFUNCTIONAL CHELATES

2.1.1 SYNTHESIS OF p-NITROBENZYLETHYLENEDIAMINETETRAACETIC ACID

The starting compound, p-nitrobenzylethylenediaminetetraacetic acid (PNB-EDTA) was synthesized by Dr. R. Gaudreault using the modified method of Yeh *et al.*[56] and was used without further characterization. All glassware used in the synthesis of the bifunctional chelates, and the subsequent protein coupling reactions, was acid washed in 50/50 nitric/sulfuric acid to remove extraneous metal ions.[145] Buffers used were either extracted with 0.01% dithizone[146] or treated by passage over Chelex 100 (BioRad) to remove various interfering cations.[147] Water was double distilled and deionized to ensure high quality, metal free solutions.

2.1.2 SYNTHESIS OF p-AMINOBENZYLETHYLENEDIAMINETETRAACETIC ACID

The p-aminobenzylethylenediaminetetraacetic acid (PAB-EDTA) was synthesized from PNB-EDTA as described by Leung [141] and is briefly outlined below.

One hundred mg of PNB-EDTA was dissolved in 50 ml of double distilled, deionized water and the pH adjusted to 11 to 12 with 1N NaOH. Thirty mg of 10% palladium on carbon (Pd/C) was added and the solution equilibrated with atmospheric hydrogen. Stirring was continued until uptake of hydrogen ceased, approximately 1 to 2 hours. The solution was filtered and the volume of filtrate reduced to approximately 2 to 3 ml by evaporating under reduced pressure. This solution was again filtered, this time through a 0.22μ Millipore filter to remove remaining catalyst. The solution was lyophilized and stored at -20°C.

2.1.3 DERIVATIZATION OF PAB-EDTA

2.1.3.1 SYNTHESIS OF p-BENZYLDIAZONIUM-ETHYLENEDIAMINETETRA-ACETIC ACID

The diazonium salt derivative of PAB-EDTA (N_2^+-B-EDTA) was synthesized according to Sundberg *et al.*.[51] Fifty mg of PAB-EDTA was dissolved in 2 ml of double distilled, deionized water to which was added 3 ml of ultrapure concentrated HCl (BDH) and kept stirring on ice. Four hundred and fifty µl of a metal free 0.5 M sodium nitrite solution or a 20% t-butyl nitrite solution was added in 50 µl increments to the stirring solution and the reaction allowed to proceed for one hour. The solution was tested on starch iodine paper and if excess nitric acid was present, a few grains of urea were added to destroy it. The solution was aliquoted into 1 ml batches and frozen at -80°C. The diazonium salt concentration was determined by the resorcinol coupling method of Koltun.[150] The procedure involved mixing a small aliquot of the reaction mixture from above with 5 ml of 0.01 M resorcinol - 0.1 M sodium acetate, pH = 4.5. Color was allowed to develop for 30 minutes and the absorbance of a suitably diluted sample was read at 385 nm. The concentration of the diazonium ions was determined from the following equation:

$$A_{385\ nm} = \epsilon_{385\ nm} \cdot b \cdot c \cdot d$$

where: $A_{385\ nm}$ = absorbance at 385 nm

$\epsilon_{385\ nm} = 21500\ M^{-1}\ cm^{-1}$

c = concentration in M

b = cell length in cm

d = dilution factor

and the overall yield determined.

2.1.3.2 SYNTHESIS OF p-BROMOACETAMIDO-BENZYLETHYLENEDIAMINE-TETRAACETIC ACID

The p-bromoacetamido-benzylethylenediaminetetraacetic acid (BrAc-B-EDTA) was synthesized according to DeReimer and Meares.[149] Approximately 240 mg of PAB-EDTA was dissolved in 1.5 ml of double distilled, deionized water and neutralized with 1 N NaOH. The solution was cooled on ice for one-half hour and 150 μl of bromoacetyl bromide was added with vigorous mixing. The solution was extracted 6 to 7 times with 6 ml aliquots of ether to remove unreacted bromoacetyl bromide and bromoacetic acid. The pH was adjusted to 2.4 and the white precipitate that formed was redissolved in water. The pH was adjusted to 2 to 3 to again form the precipitate and the procedure repeated 3 times. The final solution, adjusted to pH 2 to 3, was kept overnight at 4°C, lyophilized and stored dessicated at -20°C prior to use.

2.1.4 ANALYSIS OF BIFUNCTIONAL CHELATES

2.1.4.1 THIN LAYER CHROMATOGRAPHY (TLC)

Several different solvent systems were tested to determine the quality of both the unlabeled PAB-EDTA and radiometal chelated PAB-EDTA. Acetone, pH = 2.0 and 10% ammonium acetate : methanol (1 : 1) on silica gel have been used to determine the purity of the unlabeled compound.[141] Along with these, 70% acetonitrile, pH = 2.0 on silica gel and cellulose were used to assess purity in these studies.

In determining radiometal chelation by TLC, both silica gel and cellulose were used with the solvent systems described above as well as PEI cellulose developed in 1 M LiCl, pH = 1.0.

2.1.4.2 CHELATION WITH RADIOMETALS

The chelation reaction was carried out by first dissolving the PAB-EDTA (1.0 mg/ml) in metal free 0.1 N HCl (BDH), adding approximately 400 - 800 kBq of the required radioisotope and incubating at room temperature for at least 15 minutes prior to

TLC analysis. The radioisotopes used were injection grade $^{111}InCl_3$ (Medi-Physics) and reaction grade $^{111}InCl_3$ (AECL) and $^{67}GaCl_3$ (Frosst). Radioiodinated PAB-EDTA was also tested by TLC to determine the quality of this compound. Iodination was kindly performed by Dr. T.R. Sykes using the Iodo-gen method. Approximately 500 mg of the compound was reacted with 7.4 MBq of $Na^{123}I$ in 200 ul of phosphate buffered saline in the presence of 5 μg of Iodo-gen for 15 minutes. The radioiodinated compound was purified on preparative silica gel thin layer chromatography (TLC) using 70% acetonitrile, pH = 2.0.

2.1.4.3 *IN VITRO* STABILITY OF RADIOMETAL CHELATED PAB-EDTA

Stability of the radiometal chelated PAB-EDTA was also determined in the presence of transferrin and human serum albumin (HSA). A solution of apo-transferrin (99% iron-free, Calbiochem) at a concentration of 3 mg/ml in 0.1 M sodium citrate, pH=6.0 was mixed with 50 μg of PAB-EDTA chelated with 4 MBq of $^{111}InCl_3$. A similar solution containing 30 mg/ml HSA was also prepared and a solution of ^{111}In-PAB-EDTA with no protein served as the blank control. All solutions were incubated at 37°C and at various time intervals, 50 μl was removed for analysis by gel filtration on Sephadex G-50 and TLC on cellulose developed in methanol : 0.1 N HCl (7:3).

2.1.5 ANALYSIS OF RADIOMETALS

2.1.5.1 PURITY

THIN LAYER CHROMATOGRAPHY

TLC analysis of the ^{67}Ga and ^{111}In preparations was used to determine the presence of unwanted chelate contaminants. TLC of ^{111}In was performed on silica gel and in the case ^{67}Ga on cellulose with 10% ammonium acetate : methanol (1:1) or acetone, pH=2.0 as the solvents. The radioisotopes tested were those described above and injectable grade ^{67}Ga-citrate (Frosst).

The non-radioactive metal ion interference with radiometal binding to PAB-EDTA was assessed using the TLC systems described above. The radioisotopes tested were the reaction grade preparations of $^{111}InCl_3$ and $^{67}GaCl_3$. Levels of non-radioactive metal ions found by INAA (see below) were mixed with either $^{111}InCl_3$ or $^{67}GaCl_3$ and then incubated with 2.2 mM PAB-EDTA in 0.1 N HCl. After a 15 minute room temperature incubation, small aliquots (1 to 2 μl) were spotted on the appropriate plates and developed in 10% ammonium acetate : methanol (1:1) so that each plate contained an 8 cm strip between origin and solvent front. The developed plates were dried, cut into 10 strips and counted using a multi sample NaI(Tl) well detector (Tracor 2200) coupled to a TN-1710 multi channel analyzer. The amount of radioactivity associated with each strip was used to calculate the R_f values and percent of the radioactivity bound to PAB-EDTA.

INSTRUMENTAL NEUTRON ACTIVATION ANALYSIS (INAA)

Instrumental neutron activation analysis was used to determine the extent of contaminating non-radioactive metal ions in the various commercial preparations of ^{67}Ga and ^{111}In.[152] Samples whose original radioactivity was allowed to decay were spotted and dried on filter paper discs (Whatman #1) then sealed in polyethylene envelopes. In the case of ^{67}Ga, 2, 10 and 100 μg standards of iron, zinc, copper and titanium were prepared in a similar manner. Five, 10, 50, 75 and 100 μg standards of zinc, iron and cadmium were prepared in a identical manner for ^{111}In. Background filter discs as well as filter discs spotted with 0.1 N HCl and double distilled, deionized water were also irradiated. Irradiations were carried out at full flux (10^{12} $n \cdot cm^{-2} \cdot sec^{-1}$) for 5 minutes and, after a 14 day de-excitation period, a second irradiation for 4 hours was performed. The 5 minute irradiation included only the copper and titanium standards and the ^{67}Ga samples. After the short irradiation, samples and standards were allowed to de-excite for 1 minute and then counted for 1 minute in a 6 cm lead cave using a WIN-15 Ge(Li) Spectrometer coupled to an ND 660 multi channel analyzer. The ^{67}Ga samples were allowed to cool for 14 days then

re-irradiated for 4 hours along with the iron, zinc and cadmium standards and the ^{111}In samples. The cadmium standards and the ^{111}In samples were allowed to de-excite for 72 hours prior to counting for 15 minutes on the WIN-15 system. After a further 11 day decay period, all samples and the iron and zinc standards were counted for 10 hours on the WIN-15 system and for 2 hours on the Tracor 2200 system. The irradiations were performed at the University of Alberta SLOWPOKE Reactor Facility. Spectra analysis consisted of comparing ^{67}Ga samples to the iron, zinc, copper and titanium standards using the characteristic γ rays of ^{67}Cu, ^{59}Fe, ^{51}Ti and ^{65}Zn and comparing ^{111}In samples to the iron, zinc and cadmium standards using the characteristic γ rays of ^{59}Fe, ^{65}Zn and ^{115}Cd. Detection limits of each element were determined using the method of Currie[151] for paired observations.

2.1.5.2 RADIOMETAL PURIFICATION METHODS

ION EXCHANGE CHROMATOGRAPHY

Both ^{67}Ga and ^{111}In can be obtained in the trichloride form by using ion exchange chromatography. Using strictly controlled conditions, both isotopes can be produced without non-radioactive metal ion contamination. The procedure is based on that used by Nelson *et al.*[153] and involves the use of AG 1 x 4 or AG 1 x 8 (BioRad) ion exchange resin. The desired amount of resin, in this case approximately 2.5 ml (4g), is made into a slurry in double distilled, deionized water and poured into an acid washed column (0.5 x 13 cm). The resin is allowed to settle and the column equilibrated with the starting molarity of HCl. The isotope solution is made equivalent to the starting molarity of HCl (2 N or greater) and applied to the resin. All effluent is collected and monitored for radioactivity. Three 5 ml washes with starting HCl are used to remove non-absorbed impurities and the purified isotope is eluted with 0.1 N HCl. The first 3 ml collected after the start of elution with 0.1 N HCl represents the void volume of the column and 90 to 95% of the activity applied is collected in the next 5 ml. The column is washed with a further 20 ml of

0.1 N HCl to remove all remaining activity. The 5 ml fraction of purified isotope is concentrated by evaporating the 0.1 N HCl under a heat lamp and a stream of filtered air or nitrogen. The purified isotope is stored dry until needed, then dissolved in a small amount of 0.1 N HCl (50 to 100 μl). TLC is used to check for the presence of chelate impurities.

ETHER EXTRACTION OF RADIOGALLIUM

^{67}Ga can also be purified using a simple extraction method with di-isopropyl ether from a reducing solution.[134] The ^{67}Ga solution is dried or reduced in volume and then made 6 N with respect to HCl to a volume of 1 ml. Approximately 300 mg of silver wool is left in contact with the solution for 3 to 5 minutes at 80°C. The ^{67}Ga is extracted with 2 one ml batches of ether and washed with 1 ml of 6 N HCl. The ether is evaporated and the purified ^{67}Ga stored dry until needed, then dissolved in a small volume (50 to 100 μl) of 0.1 N HCl.

2.2 PREPARATION OF LABELED PROTEINS

2.2.1 PROTEINS ANALYZED

2.2.1.1 POLYCLONAL HUMAN IgG

Lyophilized polyclonal human IgG (poly-H-IgG) used in the labeling studies (prepared by fractionating pooled human serum to isolate the IgG component) was obtained commercially (Sigma). Prior to use, the poly-H-IgG was dissolved in 0.15 M NaCl and extensively dialyzed against 0.05 M EDTA then double distilled deionized water and lyophilized. No evidence of denaturation was observed. The lyophilized poly-H-IgG was stored at -20°C.

2.2.1.2 MURINE MONOCLONAL ANTIBODIES - MAb-M1A AND MAb-46D

MAb-46D and MAb-M1A were kindly supplied by Dr. Julia Levy (Department of Microbiology, University of British Columbia). MAb-46D is an IgG_1 generated against the CAMAL (leukemia associated) antigen. MAb-M1A is an IgG_1 generated against a methylcholanthrene induced myosarcoma of DBA-2 mice. Both MAbs were supplied in purified form, in concentrations ranging from 3 - 11 mg/ml in PBS with 0.02% sodium azide as preservative.

2.2.1.3 MURINE MONOCLONAL ANTIBODY - MAb-155H.7

MAb-155H.7 is an IgG_{2b} generated against the synthetic hapten β-D-Gal(1→3)β-D-GalNAc coupled to human serum albumin (Tβ-HSA). Ascites are produced in pristane primed irradiated Balb/c mice and harvested over a period of one to two weeks.

PURIFICATION OF MAb-155H.7

MAb-155H.7 was purified from ascites according to the following scheme on Protein-A Sepharose CL-4B (Pharmacia) (see Figure 1).

Protein-A Sepharose was prepared by swelling and washing the gel on a sintered glass filter in 0.1 M phosphate buffer, pH=7.0. A short (1cm x 9cm) column was prepared and equilibrated with starting buffer (0.05 M Tris, 0.15 M NaCl, pH=8.5).

The ascites was first centrifuged for 30 minutes at 10,000 x g and then treated in one of the following two ways prior to application to the Protein-A column:

1. A simple dilution (1:1) with starting buffer was made and the entire solution was applied with a low flow rate to the Protein-A column.
2. The ascites was first treated with a 50% then a 33% ammonium sulfate precipitation, to remove contaminating proteins such as albumin and transferrin, by diluting the ascites (1:1) with PBS and adding dropwise, with stirring, an

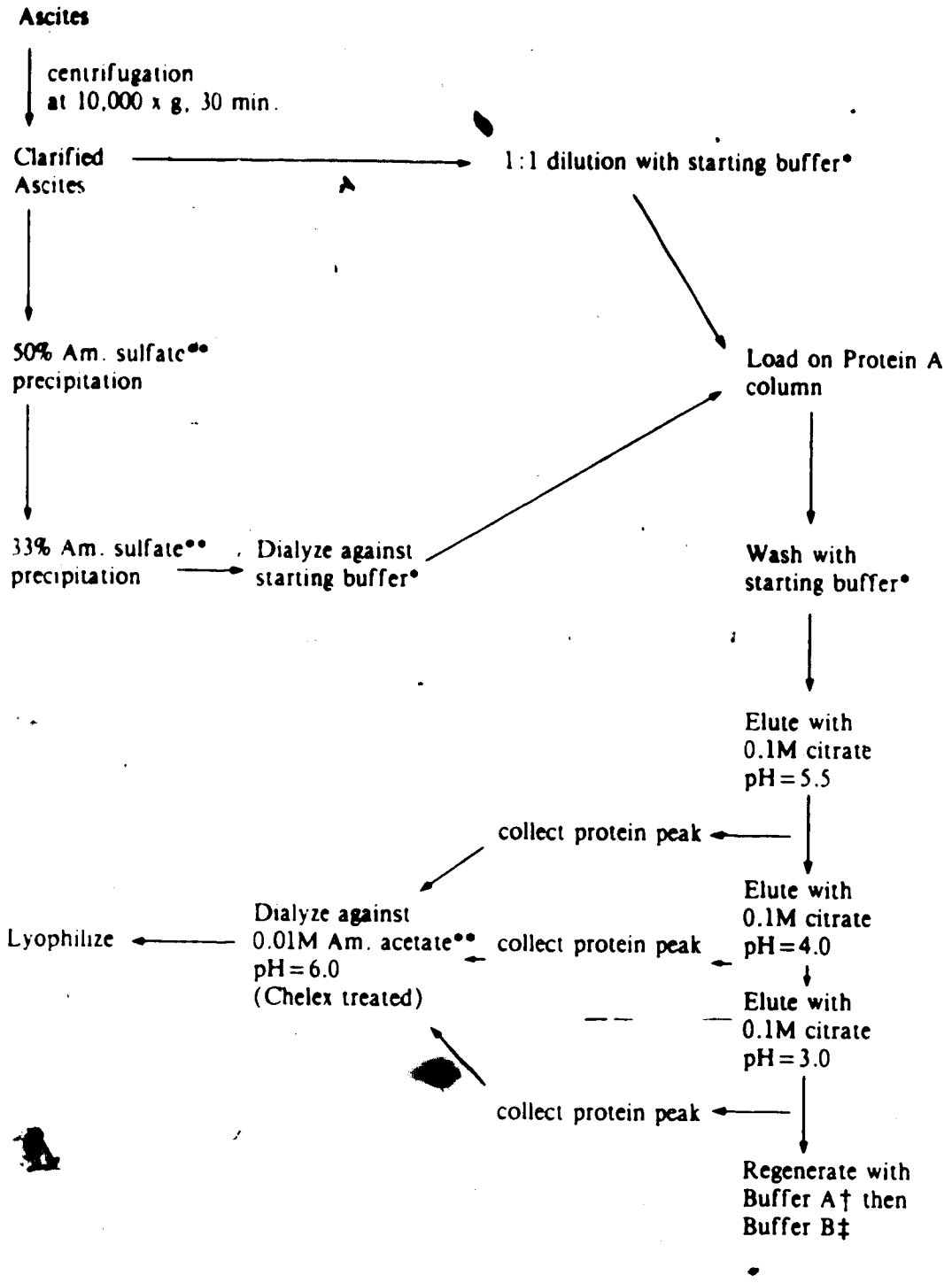


Figure 1. Purification scheme of MAb-155H.7 on Protein A Sepharose.
*starting buffer = 0.05M Tris, 0.15M NaCl, pH=8.5; **Am. sulfate = $(NH_4)_2SO_4$, Am. acetate = CH_3COONH_4; †Buffer A = 0.1M Tris, 0.5M NaCl, pH=8.5; ‡Buffer B = 0.1M CH_3COONa, 0.5M NaCl, pH=4.5.

equal volume of saturated ammonium sulfate, pH=7.0. After 2 hours at 4°C, the solution was centrifuged at 10,000 x g for 20 minutes. The precipitates were dissolved in a minimal volume of double distilled deionized water (15 to 20 ml) and extensively dialyzed against starting buffer.

The elution of the Protein-A column was monitored by a flow through 280 nm UV detector and dual pen chart recorder.

The processed ascites was applied to the column which was washed with starting buffer until the absorbance of the eluate, monitored by a flow-through UV detector (280 nm), had returned to baseline parameters. Elution of the MAb was accomplished by a step-wise gradient with 0.1 M sodium citrate, starting at pH = 5.5 then changing to pH=4.0 and finally to pH=3.0. Each protein peak collected at the three pH levels was immediately dialyzed against 0.01 M ammonium acetate, pH 6.0.

Until this point, particular care was not taken to keep the MAb preparation metal-free. However, dialysis media were treated with Chelex 100 (as previously described) and the resultant metal-free dialysate was lyophilized in acid washed vials to minimize metal ion contamination.

After elution, the Protein-A column was regenerated using 100 ml of 0.1 M Tris / 0.5 M NaCl, pH=8.5 and 100 ml of 0.1 M sodium acetate / 0.5 M NaCl, pH=4.5 consecutively. The column was then re-equilibrated with starting buffer containing 0.02% sodium azide and stored at 4°C until use.

After dialysis, each protein fraction was lyophilized, weighed and stored at -20°C.

ANALYSIS OF MAbs

PROTEIN CONCENTRATION

The protein concentration for the purified fractions of MAb-155H.7 and all subsequent protein solutions was determined using the BioRad Protein Assay kit. This procedure utilizes the wavelength shift from 465 nm to 595 nm of the

dye Coomassie Blue as it binds protein. A further modification of this assay, as described by BioRad Laboratories (Bulletin 1177), allows automated sample analysis with an ELISA plate reader equipped with a 595 nm filter. For each assay, a protein standard of IgG at concentrations of 0.05 mg/ml to 0.5 mg/ml was used to construct the standard curve. Linear regression analysis was applied to the standard curve and the unknown concentrations calculated using the linear regression parameters.

ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

Purified MAbs were tested against their respective synthetic or natural antigens in a standard enzyme-linked immunosorbent assay (ELISA). For MAb-46D, the CAMAL antigen was used as the solid phase and for MAb-155H.7, the synthetic Tβ-HSA used for the immunization protocol and containing a high hapten : HSA ratio (30 to 35) was used for initial screening. Other antigens used for MAb-155H.7 screening included the Tβ-hapten coupled to HSA (at a lower ratio than above (10 to 15)), bovine serum albumin (BSA) and Keyhole limpet hemocyanin (KLH) as well as native HSA.

All antigens were coated on 96 well microtiter plates (Dynatech) at a concentration of 1 μg / well. For CAMAL, coating was done in carbonate buffer, pH=9.6 and for Tβ-antigens, in PBS, pH=7.0. All plates were incubated overnight at 4°C and if not used within 1 - 2 days were frozen at -20°C until needed. Plates were prepared prior to use by washing once with 200 μl / well of 0.05% Tween 20 / PBS and blocking for 30 to 60 minutes with 1% BSA / PBS. Dilutions of MAb-46D were made from $1/10^2$ to $1/10^6$ (4 μg to 0.4 ng) in PBS and of MAb-155H.7 from $1/10^3$ to $1/10^6$ (2 μg to 2 ng) with serial dilutions thereafter to $1/0.33 \times 10^8$ (0.06 ng) in 1% BSA / PBS. One hundred μl of the diluted MAbs were pipetted into the appropriate wells of the microtiter plate in duplicate or triplicate. Conjugate and substrate background controls were included on each plate.

After a 1 to 1 ½ hour room temperature incubation with the test MAbs, the plates were washed 4 times with 0.05% Tween 20 / PBS (200 µl / well) and blotted dry. To test and conjugate control wells was added 100 µl of a 1/200 dilution of either rat anti-mouse IgG-AP conjugate (for MAb-46D) or goat anti-mouse IgG-HRPO conjugate (for MAb-155H.7). One hundred µl of 1% BSA / PBS was added to each substrate control well and a further one hour room temperature incubation was carried out. The wash cycle was repeated and 100 µl of p-nitrophenylphosphate in 10% ethanolamine, pH=9.8 (pNPP) substrate was added to all wells in the case of MAb-46D or 2,2'-azinodi(3-ethyl-benzthiazoline sulphonate) (ABTS) substrate (KPL) was added to all wells for MAb-155H.7. After a 20 to 30 minute incubation at room temperature to allow color development, the absorbance was measured on a Dynatech MR600 ELISA plate reader coupled to an Apple IIc computer. The computer program Immunosoft (Dynatech) was used to calculate means of replicates and to subtract substrate controls from test and conjugate control wells. Based on the protein concentration and absorbance reading at 405 nm, the antigen binding activity for each MAb was calculated and expressed as pg of MAb per absorbance unit.

SUBCLASS ISOTYPING OF MAb-155H.7

Subclass isotyping of MAb-155H.7 was determined using the Mouse Typer Sub-Isotyping kit (BioRad). This is an ELISA based technique that uses a panel of ultrapure rabbit anti-mouse reagents to determine the murine MAb class and isotype. Briefly, the MAb to be tested is incubated with its appropriate antigen and then appropriate wells are incubated with each of the following rabbit anti-mouse (RAM) reagents: RAM-IgG_1, RAM-IgG_{2a}, RAM-IgG_{2b}, RAM-IgG_3, RAM-IgM and RAM-IgA. After a 1 hour room temperature incubation, diluted goat anti-rabbit (GAR)-IgG-HRPO conjugate is added to the test wells. After a further 1 hour incubation, ABTS substrate is added and

the absorbance determined. Positive wells show absorbance readings of 2 to 3 times the negative wells. Appropriate conjugate and substrate background controls are included.

2.2.2 LABELING TECHNIQUES

2.2.2.1 METHODS OF ANALYSIS

GEL FILTRATION TECHNIQUES

Two methods of gel filtration were used in analyzing labeled proteins, conventional gel filtration and a centrifuged mini-column filtration. The gels used were BioGel P-6DG and P-100 (BioRad) and Sephadex G-50 fine (Pharmacia).

CONVENTIONAL GEL FILTRATION

For this procedure, the gel is swollen in 0.05 M EDTA, pH=6.0 and poured into a 1 x 30cm column (BioRad) to a height of 18 cm. P-6DG and P-100 are swollen by adding the dry gel (5 g of P-6DG for 38 ml of gel and 2.5 g of P-100 for 38 ml of gel) to 100 ml of 0.05 M EDTA, pH=6.0 at 90°C. The gel slurry is allowed to cool and hydrate for 1 hour. Sephadex G-50 fine was swollen by adding the dry gel (3 g for 30 ml of gel) to the EDTA buffer, boiling on a water bath for 1 hour and cooling to room temperature.

After the swelling procedure was completed, the gel slurry was washed several times with fresh EDTA buffer. The buffer was aspirated under vacuum between washings to facilitate removal of gel 'fines' that could impede column flow if left in the slurry.

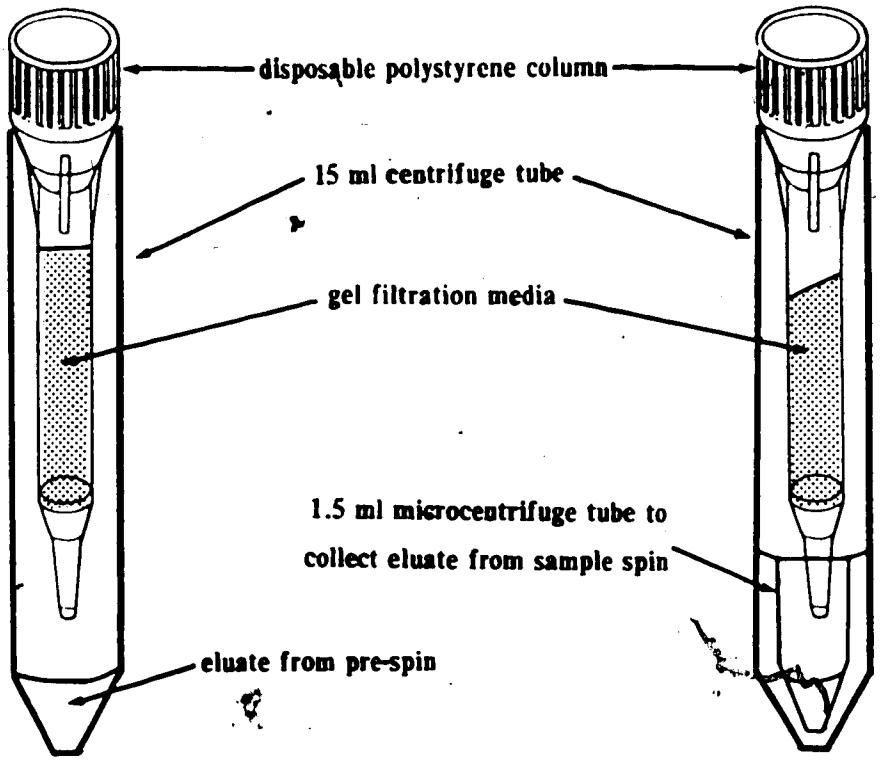
Once the column was poured it was equilibrated with 100 ml of starting buffer (0.1 M sodium citrate, pH=6.0 for BioGel P-100 and Sephadex G-50 fine and PBS, pH=7.0 for BioGel P-6DG).

The column effluent was monitored by a flow through 280 nm UV detector (LDC) and a shielded NaI(Tl) crystal with associated electronics

coupled to a dual pen chart recorder to detect protein and radioactivity content simultaneously. For preparative runs of chelate labeled protein, only the UV detector was used. Protein peaks and other peaks were collected manually as single fractions.

CENTRIFUGED MINI-COLUMN FILTRATION

This procedure, based on that described by Penefsky[15], employs a disposable 0.8 x 10 cm column (Pierce) filled with 2.5 ml of the appropriate gel (see section above). The gel is allowed to settle to a height of 5.5 cm and is equilibrated with 0.1 M sodium citrate, pH = 6.0 or PBS, pH = 7.0. The buffer is drained to the top of the gel bed and the whole column inserted into a 15 ml disposable polystyrene centrifuge tube (Falcon) (see Figure 2a). This unit is then placed in a fixed angle rotor table top centrifuge (Dynac) and centrifuged at approximately one quarter speed (20 out of 100, approximately 100 x g) for 2 minutes. One ml of buffer is removed from the gel bed and the gel height is reduced to approximately 3.5 cm. The gel itself looks shrunken and dehydrated. The column is then transferred to a new 15 ml centrifuge tube containing a 1.5 ml polypropylene micro-centrifuge tube (Eppendorf) with the cap removed (see Figure 2b). One hundred to 300 μl of the protein solution to be analyzed is pipetted onto the gel bed. The apparatus is re-centrifuged as described above and the purified protein collected in the micro-centrifuge tube. The columns can then be washed with 100 μl of starting buffer 2 to 5 times with new micro-centrifuge tubes used to collect each wash. Protein content is assessed by the BioRad protein assay (described previously) or recovery of radioactively labeled standard proteins. Initial testing of this procedure assessed time and centrifuge speed on protein recovery and breakthrough of unreacted bifunctional chelate, radiometal and radioiodine.



A) APPARATUS FOR PRE-SPIN

B) APPARATUS FOR SAMPLE COLLECTION

Figure 2. Mini-column apparatus used for centrifuged gel filtration procedure.

THIN LAYER CHROMATOGRAPHY (TLC)

Thin layer chromatography (TLC) was used as the major analytical technique to determine:

1. the number of chelate molecules attached per protein molecule and as a quick analytical method for radiometal binding to purified chelate labeled protein and
2. as a method for assessing non-radioactive metal ion interference with ^{111}In chelation by labeled proteins.

For determining labeled chelate ratios in reaction mixtures, the following procedure was used. A standardized 20.17 mM $InCl_3$ solution was prepared from crystals of ultrapure $InCl_3$ (Aldrich) accurately weighed and dissolved in ultrapure 0.1 N HCl (BDH). From this solution, further dilutions were made in ultrapure 0.1 N HCl so that a 2 : 1 ratio of $InCl_3$ to chelate present in 5 μl of the reaction mixture was produced in 5 μl. All dilutions of the standard $InCl_3$ were made based upon actual reaction parameters and were performed just prior to the assay. To this diluted standard $InCl_3$ was added 37 to 74 MBq of no-carrier-added $^{111}InCl_3$ (AECL). The number of radioactive atoms present in this amount of $^{111}InCl_3$ was not enough to alter the calculations for the ratios. This 5 μl aliquot of standard $InCl_3$ was mixed with 5 μl of 0.1 M sodium citrate, pH = 6.0, 15 minutes prior to the assay. At the time of assay, 5 μl of the reaction mixture was removed and diluted with 5 μl of 0.1 M sodium citrate pH = 6.0 and incubated for 15 minutes with the 10 μl of diluted standard $InCl_3$ solution.

After incubation, 1 to 2 μl of this mixture was spotted on cellulose or silica gel TLC plates and developed in the appropriate solvent system as described above. The radioactivity of each plate was quantified by either a TLC Linear Analyzer (Berthold) coupled to Canberra Series 80 multi channel analyzer and a single pen chart recorder or by cutting each plate into 10 strips and counting the strips in a multi sample NaI(Tl) detector (Beckman). R_f values and percent radioactivity bound to the protein were calculated to determine the yield of the bifunctional chelate

reaction.

For determining binding of no-carrier-added radiometal to purified, chelate-labeled protein a procedure similar to that described above was used. A 100 μl aliquot of chelate-labeled protein in 0.1 M sodium citrate, pH=6.0, was mixed directly with the no-carrier-added $^{111}InCl_3$ or $^{67}GaCl_3$ in 0.1 N HCl. The amount of radioactivity ranged from 1.85 MBq to 74 MBq depending upon the specific activity required. The volume of acidic radiometal added did not affect the pH of the 100 μl aliquot of protein in 0.1 M sodium citrate, pH=6.0. Alternatively, the radiometal was first mixed with an equal volume of 0.1 M sodium citrate, pH=6.0 and then incubated with the chelate-labeled protein prior to the TLC assay. For this particular assay, only cellulose plates developed in methanol : 0.1 N HCl (7:3) were used. Determination of R_f values and percent radioactivity bound to the protein was identical to that described above.

For determining non-radioactive metal ion interference with no-carrier-added radiometal chelation to chelate-labeled poly-H-IgG, the following procedure was used.

Appropriate molar ratios of $ZnCl_2$ or $FeCl_3$ to chelate-labeled poly-H-IgG ranging from 0.1:1 to 100:1, were mixed with $^{111}InCl_3$ or $^{67}GaCl_3$ in 0.1 N HCl. Each radiometal solution was then diluted with an equal volume of 0.1 M sodium citrate buffer, pH=6.0 and incubated with 65 μM poly-H-IgG-B-EDTA in the same citrate buffer at room temperature for 15 minutes. TLC was performed on cellulose with methanol : 0.1 N HCl (7:3) as the solvent and developed plates analyzed for radioactivity as described above.

IN VITRO RADIOMETAL CHELATE-LABELED PROTEIN STABILITY

The *in vitro* stability of radiometal chelate labeled protein in solution was tested over a period of three weeks at three temperatures. Three 50 μl solutions of chelate labeled poly-H-IgG containing approximately 5 chelate groups / molecule were labeled with 5.5 MBq of $^{111}InCl_3$. The initial incubation period was 20 minutes

at room temperature for all mixtures to determine baseline parameters. TLC was performed in duplicate on cellulose plates developed in methanol : 0.1 N HCl (7:3). The solutions were incubated at 4°C, 25°C (room temperature) and 37°C respectively and analyzed daily for 3 days, every 2 days to 1 week and once at 3 weeks.

2.2.2.2 CHELATE LABELING REACTION CONDITIONS

N;-B-EDTA COUPLING REACTION

Murine MAb-46D and MAb-M1A, 3 to 11 mg/ml in PBS containing 0.02% sodium azide, were dialyzed extensively against 0.15 M NaCl (Chelex 100-treated). Poly-H-IgG was dissolved directly in the 0.15 M NaCl (Chelex 100-treated) at a concentration of 20 mg/ml. Protein concentrations were determined before and after the addition of the diazonium salt of PAB-EDTA (N;-B-EDTA) using the BioRad assay. The diazonium salt concentration was determined prior to the coupling reactions using the resorcinol assay and N;-B-EDTA was added at concentrations that ranged from 10:1 to 50:1 N;-B-EDTA : protein.

Typically, 1 ml of the protein solution was transferred to a 5 ml acid washed glass vial containing a micro spin bar. Fifty to 200 µl of N;-B-EDTA was added to the protein solution with stirring in 10 to 20 µl aliquots. The pH was monitored and re-adjusted between chelate additions with metal-free 5 N NaOH to pH=9.0. The final solution was transferred to an acid washed polypropylene micro centrifruge tube (Eppendorf) and centrifuged at 15,000 x g for 1 minute to remove precipitated protein.

The reaction was allowed to proceed with stirring at 4°C overnight. The standardized $^{111}InCl_3$ TLC assay was performed on a 5 µl aliquot of the reaction mixture to determine percent chelate labeling and unreacted chelate was removed by gel filtration on BioGel P-100 equilibrated with 0.1 M sodium citrate, pH=6.0 as described previously. Purified, chelate-labeled protein was aliquoted into 100 to 200 µl batches and stored at -80°C.

BrAc-B-EDTA COUPLING REACTION

Poly-H-IgG or MAb-155H.7, purified and lyophilized, was dissolved at a concentration of 15 to 20 mg/ml in 0.2 M phosphate buffer, pH=8.5. Initial testing of various strengths of phosphate buffer at pH=8.5 was carried out to choose the strongest buffering capacity with the least damage to the protein. Three solutions of poly-H-IgG were made in 0.01 M phosphate buffer, pH=8.5 and enough 2.0 M phosphate, pH=8.5 was added to produce 0.2, 0.5 and 1.0 M phosphate solutions at 1.5, 5 and 23 mg/ml of poly-H-IgG. Stock protein solutions were made to the same concentrations as above by adding 0.01 M phosphate, pH=8.5 instead of the concentrated phosphate solutions to serve as controls. The solutions were well mixed and then centrifuged for 1 minute at 15,000 x g to remove precipitated protein. Protein concentration was determined using the BioRad protein assay.

Coupling reactions were based on 2.0 mg (1.25×10^{-5} moles) of protein and these were usually contained in 100 to 150 μl of 0.2 M phosphate buffer, pH=8.5. The BrAc-B-EDTA was weighed directly into the reaction tube or dissolved in metal free 0.1 N NaOH. The dissolved BrAc-B-EDTA was pipetted into the reaction vial and lyophilized before use. This procedure was used for those reactions requiring less than 1 mg of BrAc-B-EDTA as these amounts were difficult to weigh accurately. Ratios of 1:1, 10:1, 50:1 and 100:1 of BrAc-B-EDTA : protein were tested. To start the reaction, the protein solution was added to the required BrAc-B-EDTA and the reaction proceeded for 2 hours at 37°C. No adjustment of pH was necessary.

In the case of MAb-155H.7, identical reactions omitted the BrAc-B-EDTA to determine the effect of the reaction conditions (0.2 M phosphate, pH=8.5 and 37°C) on the *in vitro* binding of the MAb to its synthetic antigen.

At the end of reaction time, a 5 μl aliquot was tested by the standard $InCl_3$ TLC assay to determine the percent bifunctional chelate binding to the protein. The reaction mixture was purified by gel filtration on BioGel P-100 in 0.1 M sodium citrate, pH=6.0 and the purified protein stored at -80°C in 100 to 200 μl aliquots.

For both bifunctional chelate derivative labeling techniques, final protein concentrations were determined and the standard TLC assay on cellulose only was used to periodically check the number of chelate groups per protein molecule.

IODINATION REACTION CONDITIONS

Three methods of iodination were tested for their effects on poly-H-IgG, MAb-46D and MAb-155H.7. For all methods used, proteins were dissolved or dialyzed into 0.01 M PBS, pH = 7.0. One hundred μg of protein contained in 20 to 100 μl of PBS was used for each technique. All experiments were performed in duplicate. Removal of unbound radioiodine utilized conventional gel filtration on BioGel P-6DG in PBS or centrifuged mini column chromatography on BioGel P-100, P-6DG or Sephadex G-50 fine in PBS. Assay for unreacted radioiodine in purified protein fractions was determined by a standard trichloroacetic acid (TCA) precipitation. The procedure is based on the premise that only covalently bound radioiodine will precipitate with the protein.[21] Typical assay conditions involved the use of a carrier protein (0.5 ml of 1% BSA / PBS) and carrier NaI (5 to 10 μl of 1 M NaI). To this was added 2 to 5 μl of the purified iodinated protein, the solution mixed well and 0.5 ml of 20% TCA in water added as the final step. After vigourous vortexing, the sample is centrifuged for 1 minute at 15,000 x g to pellet the precipitated protein. The supernatant is removed and both pellet and supernatant are assayed for radioactivity in a multi sample NaI(Tl) detector (Beckman) equipped with an automatic program to calculate ^{125}I dpm. Percent radioacitivity precipitated is then calculated.

IODINE MONOCHLORIDE LABELING

To minimize protein damage and non-radioactive iodine incorporation, a 1:1 ratio of iodine monochloride (ICl) : protein was chosen. A working solution of 10 μg/ml of ICl was prepared by diluting a stock solution of ICl (4 mg/μl, Aldrich) with 2 N NaCl. To 100 μg of MAb-155H.7 in 60 μl of PBS was added

50 μl of a stock solution of $Na^{125}I$ (40 MBq/ml) in 0.5 M phosphate buffer, pH = 7.0. The reaction was started by adding 10 μl of diluted ICl and continued for 30 seconds with gentle agitation. The reaction was stopped by adding 60 μl of 0.1 M $Na_2S_2O_5$ and total radioactivity was measured prior to removal of unreacted ^{125}I by gel filtration on BioGel P-6DG. The column was monitored by the flow through UV detector and the NaI(Tl) crystal as previously described and the iodinated protein and unreacted ^{125}I were collected as single peaks. TCA precipitation of the protein peak and the total recovered ^{125}I were used to calculate purity and the percent ^{125}I incorporation.

CHLORAMINE-T LABELING

A significant excess of chloramine-T (CL-T) to protein was used in this experiment (73:1 on a molar basis of CL-T : protein). A 2 mg/ml solution of CL-T in PBS was freshly prepared just prior to iodination. The stock solutions of MAb-155H.7 and $Na^{125}I$ were identical to those used for ICl labeling (100 μg of MAb-155H.7 and 50 μl of the 40 MBq/ml $Na^{125}I$ solution). The reaction was started by the addition of 5 μl (10 μg) of the CL-T solution and continued for 30 seconds with gentle agitation. The reaction was terminated with 60 μl of 0.1 M $Na_2S_2O_5$ and purified as described above. All calculations were as described.

IODO-GEN LABELING

For this set of experiments, 1, 3 and 5 μg of Iodo-gen per 100 μg of MAb-155H.7 were tested in duplicate for reaction times of 5, 10 and 30 minutes. For 125 μg of MAb-46D, 8 μg of Iodo-gen was used for a reaction time of 30 minutes only. The required amount of Iodo-gen was plated onto the bottom of 12 x 75 mm disposable glass culture tubes (Fisher) from a 1 mg/ml solution of Iodo-gen in chloroform. The chloroform was evaporated with a gentle stream of filtered air or nitrogen. The reaction was initiated by adding the protein (100 μg

of MAb-155H.7 (as described above) or 125 μg of MAb-46D) to the reaction tube followed immediately by 50 μl of the stock $Na^{125}I$ solution (as above). At the end of reaction, the solution was transferred to a 1.5 ml polypropylene micro centrifuge tube containing 20 μl of 1 M NaI. After a 15 minute incubation with the cold NaI, the entire mixture was assayed for radioactivity and unreacted ^{125}I removed as described above. Purity was checked by TCA and percent bound to protein calculated as described above.

2.2.3 *IN VITRO* TESTING OF LABELED PROTEINS

2.2.3.1 ENZYME-LINKED IMMUNOSORBENT ASSAYS

Labelec MAbs were tested before and after radiolabeling for retention of *in vitro* immunoreactivity in a standard enzyme-linked immunosorbent assay (ELISA). Bifunctional chelate labeled MAbs were tested before and after labeling with ^{111}In as well. The standard ELISA described previously was employed for all labeled MAbs. Comparison of the labeled MAbs to unlabeled MAb was included in every test and the percent of retained binding for these MAbs was determined from the unlabeled MAb binding.

2.2.3.2 WHOLE CELL ELISA

A standard ELISA using cells grown in or attached to 96 well microtiter plates was used to screen unlabeled and radioiodine- or bifunctional chelate-labeled MAb-155H.7 for binding to both murine and human cancer cell lines. The test cell lines included the following murine lines:

1. EMT-6, a murine mammary sarcoma of Balb/c mice.[156]
2. RI, a radiation induced lymphoma of CBA mice.[157]
3. EL4, a 9:10-dimethyl-1:2-benzanthracene induced lymphoma of A strain mice.[158]

and the following human cell lines:

1. LoVo, a CEA secreting colorectal adenocarcinoma.[159]

2. SW1116, another CEA secreting colorectal adenocarcinoma.[140]
3. MIA-PACA, a pancreatic tumour line maintained in Dr. Longenecker's lab (Department of Immunology, University of Alberta).

For the adherent cell lines (EMT-6, LoVo, MIA-PACA and SW1116), cells were innoculated at 1 x 10^4 cells per well in sterile 96 well microtiter plates (Nunc) and allowed to grow for 1 to 2 days to produce a monolayer in the bottom of each well (approximately 5 x 10^4 to 1 x 10^5 cells per well). For the suspension cell lines (EL4 and RI), an alternate technique was used to fix the cells to the microtiter plate. In this case, 100 μl per well of a 50 μg/ml solution of poly-L-lysine in PBS is incubated on the 96 well plate overnight at 4°C. The plate is washed once with 200 μl per well with PBS and 100 μl per well of 0.1% glutaraldehyde in 0.1 M $NaHCO_3$ is added to the plate for a further 3 hour incubation at room temperature. After another wash cycle with PBS, approximately 1 x 10^5 cells per well are added to the plate, the plate is centrifuged at 1000 rpm for 4 minutes and incubated at 37°C for 1 hour. At this point, the adherent and non-adherent cells are treated identically. The supernatant is removed and replaced with 200 μl per well of 0.05% glutaraldehyde in PBS for a 3 to 5 minute room temperature incubation. The glutaraldehyde is removed, replaced with 200 μl per well of 5% fetal bovine serum (FBS - heat inactivated (Gibco)) and incubated overnight at 4°C. Prior to use, the plates are washed once with 0.05% Tween 20 in PBS and test MAbs are added to the appropriate wells. From this point, the assay is identical to that described above.

The proteins tested included unlabeled MAb-155H.7 and poly-H-IgG as well as 'mock' iodine and 'mock' indium-labeled MAb-155H.7 'Mock' iodination was performed using the Iodo-gen technique with replacement of the $Na^{125}I$ solution with 10 μl of 0.1 N NaOH in 100 μl of 0.5 M phosphate buffer, pH = 7.0 with treatment of the MAb as described for this technique. 'Mock' indium labeling involved the substitution of 0.1 N HCl for the $^{111}InCl_3$ solution and was performed on three levels of chelate-labeled MAb-155H.7. These included preparations of the MAb that contained 0.2, 0.6 and 7 chelate groups per protein molecule. Protein assays were determined as previously

described and ng of protein per absorbance unit for MAb-155H.7 and poly-H-IgG were calculated for each cell line for labeled and unlabeled protein.

2.2.3.3 LIVE CELL UPTAKE

^{125}I- and ^{111}In-labeled MAb-155H.7 was also screened against live cell preparations of the murine cell lines used in the fixed cell ELISA including the murine mammary adenocarcinoma, TA3/Ha.[141]

Uptake was performed on 1 x 10^6 cells in suspension in RPMI 1640 cell culture media (Gibco) both with and without 10% FBS. The adherent cell line, FMT-6, was trypsinized just prior to the assay to produce a cell suspension. Cells were tested by trypan blue exclusion to determine viability. MAb-155H.7 and poly-H-IgG were iodinated with Iodo-gen and the chelate labeled MAb-155H.7 containing 0.2, 0.6 and 7 chelate groups per molecule was labeled with $^{111}InCl_3$. Quality control of labeled preparations was performed prior to the uptake study and included TCA precipitation, TLC and a standard ELISA. Labeled proteins were diluted in 1% BSA in PBS or in PBS alone. $^{111}InCl_3$ and NaI(Tl) were included as uptake controls for chelated and iodinated proteins respectively.

One hundred μl (3 or 30 ng) of the labeled protein was added to 0.5 ml of RPMI 1640 media containing 1 x 10^6 cells. The cells were incubated for one hour in a 37°C shaking water bath. The cells were centrifuged at 1100 rpm for 5 minutes and the cell pellets washed twice with RPMI 1640 media. Radioactivity was assayed in both pellets and supernatants in a multi sample NaI(Tl) detector (Beckman) and percent uptake per 10^6 cells calculated. Uptake conditions using incubation media with and without FBS and BSA was determined using TA3/Ha cells only.

2.2.3.4 RADIOACTIVE BINDING ASSAY (RBA)

Radioiodine- and radiochelate-labeled MAb-155H.7 were assessed for their ability to bind to various forms of the T-antigen in a radioactive binding assay (RBA) using Tβ-HSA and commercially available forms of the synthetic T-antigen, Tα-synsorb (Tα-S) and Tβ-synsorb (Tβ-S) (Chembiomed). Synsorb is an inert, insoluble matrix to

which is coupled either the Tα or Tβ complex. As a nonspecific control for the T-synsorb experiments, B-synsorb, the insoluble matrix without any attached antigen, was used.

Immulon Remova-well strips (Dynatech) were coated overnight with 1 μg per well of Tβ-HSA in PBS. Prior to addition of the radiolabeled MAb-155H.7 and radiolabeled poly-H-IgG, all wells were 'blocked' with 1% BSA/PBS to prevent non-specific adherence of radiolabeled proteins to the wells. Approximately 0.5 to 1.0 mg of Tα-S (0.27 μmoles of Tα per g of synsorb), Tβ-S (0.58 μmoles of Tβ per g of synsorb), or B-S were weighed into microcentrifuge tubes (Eppendorf) and 'blocked' with 0.5 ml of 1% BSA/PBS. The amount of Tβ and Tα was determined for each tube from the amount stated on the label and the results expressed in terms of this amount. Radiolabeled protein solutions used for the live cell uptake were also used here with experiments conducted in duplicate or triplicate and a standard ELISA run in parallel for comparative purposes. All incubations were for one hour at room temperature. Tβ-HSA coated wells and all synsorbs were washed twice with PBS prior to analysis for radioactivity. Radioactive counting was performed using a multi-sample NaI(Tl) detector (Beckman) and the amount of MAb-155H.7 or poly-H-IgG bound to Tβ-HSA and the various synsorbs calculated.

2.2.3.5 HISTOLOGICAL SCREENING

Histological screening of unlabeled and both 'mock' iodine- and 'mock' indium-labeled MAb was performed in Dr. Willan's laboratory (Department of Pathology, Edmonton General Hospital). The MAbs were screened against a panel of human adenocarcinoma tissue sections. The frozen tissue sections were mounted on glass slides and incubated with the primary antibody (i.e. labeled or unlabeled MAb-155H.7) for 30 minutes. After washing, the slides were fixed in 0.05% glutaraldehyde in PBS, washed again and incubated with the second antibody (biotinylated horse anti-mouse IgG) for 30 minutes. The wash cycle was repeated, the slides were incubated with premixed ABC reagent (Avidin-Biotin HRPO, Vectastain) for 60 minutes, washed again and incubated 5 minutes with the substrate (diaminobenzidine tetrahydrochloride

(DAB). The slides were then counterstained with hematoxylin.

2.2. *IN VIVO* TESTING OF LABELED PROTEINS

2.2.4.1 BIODISTRIBUTION STUDIES

Biodistribution studies were performed in 2 to 3 month old normal Balb/c mice and CAF_1 (first generation cross of Balb/c and A strain mouse) mice carrying the TA3/Ha adenocarcinoma as a subcutaneous tumour. Mice were maintained on standard laboratory rodent chow and water *ad libitum*. Tumours were implanted by injection of 10^6 TA3/Ha cells into the right flank of the appropriate strain of mouse. Approximately 7 days were required for appearance of the TA3/Ha tumour. Rapid growth of the tumour was observed thereafter. Biodistribution was performed at 6, 24, 48 and 72 hours post injection for labeled proteins and at 15 min, 1, 6 and 24 hours post injection for labeled PNB-EDTA. The following preparations were screened in normal Balb/c mice: $Na^{125}I$, ^{111}In-citrate, ^{111}In-PNB-EDTA-^{125}I, ^{111}In-poly-H-IgG and ^{125}I-poly-H-IgG. ^{131}I-MAb-155H.7 and ^{111}In-MAb-155H.7 were compared to ^{125}I-poly-H-IgG in TA3/Ha tumour-bearing mice. All iodinations were performed using Iodo-gen as previously described. Poly-H-IgG and MAb-155H.7 were labeled using BrAc-B-EDTA. Quality control was performed on all MAbs prior to injection.

Dual-labeled counting was used for most experiments to expedite results and in the case of the comparison of ^{131}I- and ^{111}In-MAb-155H.7 with ^{125}I-poly-H-IgG, triple label counting on the Tracor 2200 multi sample NaI(Tl) detector with an automatic crossover correction program for three isotopes (C. Ediss, Faculty of Pharmacy, see Appendix 2) used to identify the various components. Labeled preparations were administered by I.V. injection through a tail vein and the animals were sacrificed by CO_2 euthanasia at the appropriate time intervals. Blood was collected by heart puncture and all organs were dissected, blotted and weighed prior to counting. In the case of the thyroid, a section of esophagus, trachea and associated tissue was removed along with the thyroid due to the extemely small size of this organ in mice.

Dual-labeled samples were counted on the Beckman multi sample NaI(Tl) detector and automatic crossover correction of the high and low energy spillover of ^{111}In into ^{125}I. The triple label crossover program automatically corrected the high energy spillover of ^{111}In into the ^{131}I channel, of ^{131}I into the ^{111}In channel and of the spillover of both high and low energy spillover of ^{111}In and ^{131}I into the ^{125}I channel by using appropriate standards of each isotope. Details of this program are given in Appendix 2.

The injected dose was calculated from an accurate weight of the material injected and an accurate weight of a 1 / 20 or 1 / 50 dilution of the injection mixture assayed in the NaI(Tl) dectector systems described above.

For all distribution studies, percent dose per organ, percent dose per gram (except for thyroid and carcass) and tissue : blood ratios were calculated for each organ. As well, the percent dose remaining was calculated for each animal. An average of 4-5 animals was used per time period.

2.2.4.2 GAMMA CAMERA SCINTIGRAPHIC STUDIES

Imaging was performed on TA3/Ha tumour-bearing mice injected with ^{111}In-MAb-155H.7, ^{131}I-MAb-155H.7, ^{131}I-poly-H-IgG or ^{111}In-citrate (1.85 MBq of ^{111}In or 4 MBq of ^{131}I). Images were taken at 24, 48, 72 and 96 hours with a Pho-Gamma IV gamma camera (Searle) interfaced to an ADAC computer and the animals were dissected at the end of scanning to determine actual percent dose per organ, percent dose per gram and tissue : blood ratios of the respective compounds. Image counts were computer normalized to the same count rate for all time periods in order to provide comparative data between ^{111}In and ^{131}I.

3. RESULTS AND DISCUSSION

3.1 PREPARATION OF BIFUNCTIONAL CHELATES

3.1.1 SYNTHESIS OF PNB-EDTA AND PAB-EDTA

The synthetic scheme for PNB-EDTA and PAB-EDTA as supplied by Dr. R. Gaudreault is shown in Figure 3. This method represents a simplified procedure compared to that first published by Sundberg *et al.*.[6] The preparation of the bifunctional chelating agents requires specific attention to the treatment of glassware and buffers to ensure that extraneous metal ion contamination does not occur, especially after the final purification step to isolate PNB-EDTA. The ion exchange system used to purify PNB-EDTA utilizes a formic acid gradient which is sufficient to dissociate most metal ion chelate complexes that may be formed during synthesis. Removal of extraneous metal ions is generally accomplished by a mixed acid wash in 50:50 nitric:sulfuric acid for all glassware and acid resistant plasticware. Non-acid resistant plasticware can be treated in a 0.01 M Na_2EDTA bath followed by thorough rinsing with double distilled deionized water. All buffers should be treated by extraction with a 0.01% dithizone solution or by passage through a Chelex 100 column.

From practical experience obtained during these experiments, the preliminary steps mentioned above proved to be invaluable in obtaining high ^{111}In labeling yields to chelated proteins.

3.1.2 DERIVATIZATION OF PAB-EDTA

Two derivatives of PAB-EDTA (N;-B-EDTA and BrAc-B-EDTA) were successfully prepared and characterized. The reaction scheme for both compounds is outlined in Figure 3.

The diazotization reaction was accomplished by using two different reagents, t-butyl-nitrite and sodium nitrite. Sodium nitrite is the reagent of choice both in Sundberg's studies[6] as well as Yeh's studies.[32] Two reactions with sodium nitrite produced overall yields of 36.5 and 37.4%, whereas two reactions performed with t-butyl-nitrite produced overall

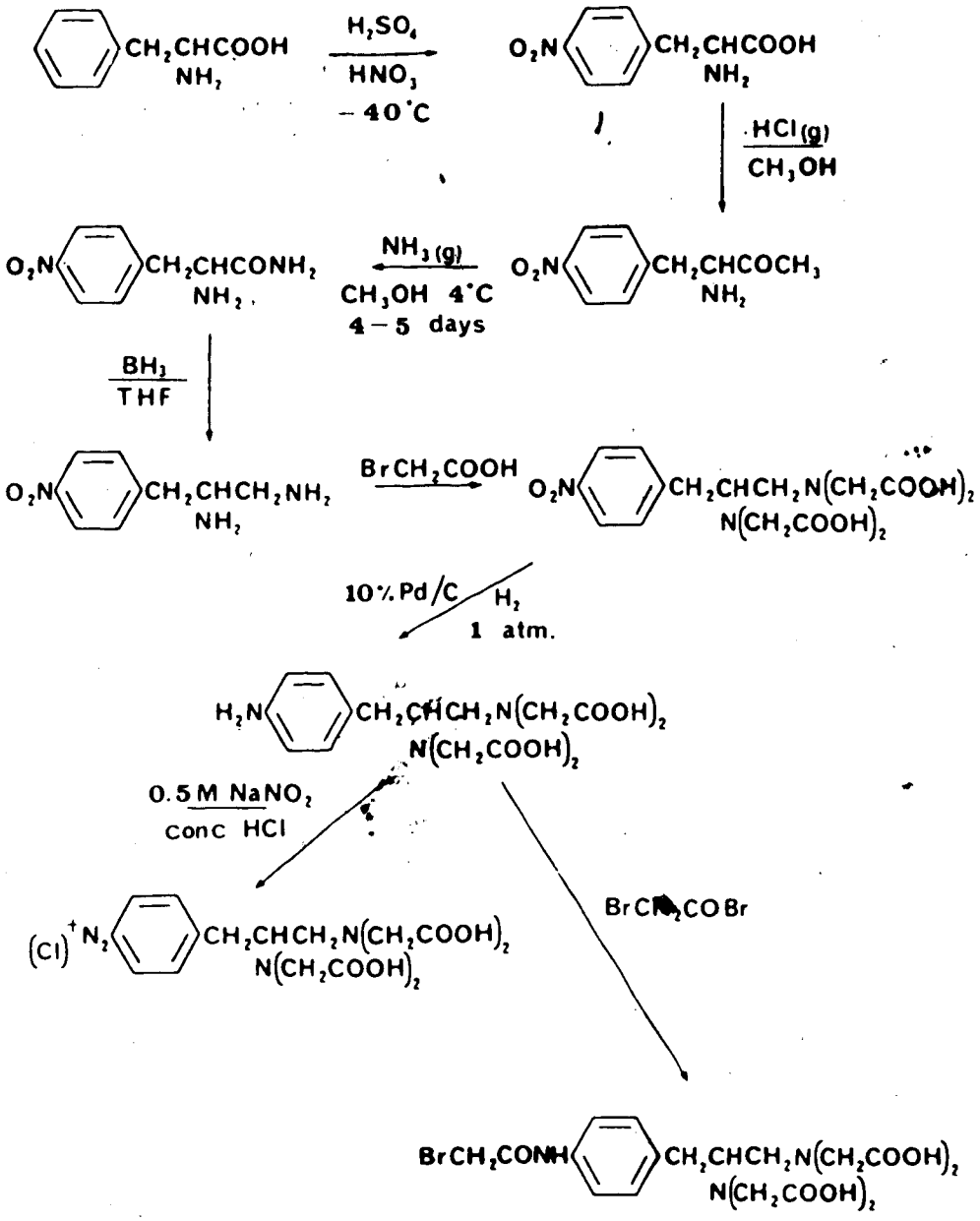


Figure 3. Synthetic scheme for the preparation and derivatization of PNB-EDTA.

yields of 16.7 and 32.5%. The two reagents gave comparable yields and the variability seen with the t-butyl-nitrite can be attributed to the instability of t-butyl-nitrite solutions upon prolonged storage. For this reason, fresh solutions should be used whenever possible.

A standard method of determining the concentration of diazonium salt present in the bifunctional chelating solution was used, namely, coupling to resorcinol.[150] This technique was initially applied to determination of the concentration of p-carboxyphenylazoinsulins in solution. As the diazonium compound reacts with resocinol, it forms a highly colored complex with an absorption maximum at 385 nm. The molecular extinction coefficient of this colored compound has been determined to be 21,500[150] and the concentration can be calculated from the equation:

$$OD_{385\ nm} = \epsilon_{385nm} \cdot c \cdot b \cdot d$$

where:

$OD_{385\ nm}$ is the optical density at the maximum wavelength of absorption, 385 nm; $\epsilon_{385\ nm}$ is the molecular extinction coefficient, 21,500; c is the molar concentration; b is the optical path length; and d is the dilution factor.

The N;-B-EDTA solution is highly acidic and to maintain stability should be stored at -80°C. Short term stability studies of the N;-B-EDTA at room temperature for time intervals of 10, 30, 60 and 90 minutes indicated no breakdown of the compound over the time period studied. As well, long term stability was monitored over several months at -20°C and -80°C. The compound had lost 4% and 12% of its diazo functionality at -20° after two and three months, respectively. However, analysis of the compound stored at -80°C showed the compound had lost only 5% of its diazo functionality after 10 months.

The BrAc-B-EDTA was prepared by the method of DeReimer and Meares.[149] The overall yield was 29% and produced a [redacted]ly yellow colored, fluffy powder that was stable for several months when stored dessicated at -20°C.

3.1.3 ANALYSIS OF BIFUNCTIONAL CHELATES

3.1.3.1 THIN LAYER CHROMATOGRAPHY (TLC)

A variety of TLC systems were used to assess the purity of the non-radioactive compounds synthesized and the radiometal chelated compounds. The N;-B-EDTA was not tested on TLC due to the instability of the diazo group in the solvent systems used here. This compound gave consistently erratic results and the spots observed on TLC were probably due to breakdown products of the N;-B-EDTA as well as the unreacted PAB-EDTA that remained in solution. At the end of the dervatization reaction, no attempt was made to remove the unreacted PAB-EDTA because of the instability of the diazo group.

When PNB-EDTA, PAB-EDTA and BrAc-B-EDTA were chromatographed on silica gel, developed in 10% ammonium acetate : methanol (1:1), R_f values of 0.85, 0.9 and 0.9 respectively, were obtained. These correspond well with the values reported by Leung[141] for these compounds using this system. Visualization of the compounds was accomplished by spraying the plates with ninhydrin reagent for PNB-EDTA and PAB-EDTA or with p-nitrophenylpyridine reagent for BrAc-B-EDTA. Other systems were also developed and these results are shown in Table 5. The acidic acetone solvent used by Leung[141] and the acidic acetonitrile developed here both showed R_f values less than 0.5 while all other systems had R_f values between 0.7 and 0.9 for PAB-EDTA.

These systems were also used to determine the extent of radiometal chelation to PAB-EDTA and those that generated R_f values greater than 0.5 were more useful for discriminating between radiometal chelate and unreacted radiometal. All results obtained are summarized in Table 5.

From the data in Table 5, it is apparent that two different stationary phases (silica gel and cellulose) are requir[illegible]ding upon the isotope being used. For ^{111}In, the silica gel system with eith[illegible]m acetate : methanol (1:1) or 70% acetonitrile, pH=2.0 solvent sat[illegible]tes unreacted ^{111}In from chelated ^{111}In. However, when the cellulose system is used with the same solvents, good resolution is not

TABLE 5. R_f Values of Radiometals and their PAB-EDTA Complexes in Various TLC Systems.

Compound	TLC System*				
	70% AcCN, pH=2 Silica Gel	70% AcCN, pH=2 Cellulose	10% AmAc:MeOH (1:1) Silica Gel	105 AmAc:MeOH (1:1) Cellulose	1M LiCl, pH=1 PEI Cellulose
^{67}GaCl$_3$	0	0.29	0	0.13	0.08
^{111}InCl$_3$	0	0.11	0.17	0.70	0.04
PAB-EDTA**	0.40	0.65	0.90	0.70	0.70
^{67}Ga-PAB-EDTA	0.03	0.52	0.06	0.74	0.76
^{111}In-PAB-EDTA	0.42	0.24	0.77	0.70	0.70
^{67}Ga-EDTA	-	0.70	-	0.70	0.95
^{123}I-PAB-EDTA	-	0.40	-	0.72	0.60

* AcCN = acetonitrile; AmAc = ammonium acetate; MeOH = methanol; PEI = polyethylimine

** visualized with ninhydrin reagent

obtained and these two species cannot be differentiated. In the case of ^{67}Ga the exact opposite is true, where cellulose separates the chelated species from the unreacted species but silica gel does not. This might be explained by the fact that the silica gel has binding sites for the unreacted indium and effectively holds this species at the origin. This attraction is greatly intensified for gallium and is strong enough to dissociate the gallium from the chelate complex. In the cellulose system no such force exists and this allows the chelated gallium to remain with the chelate. As well, unreacted gallium is not as soluble as the unreacted indium in the solvents used, especially in the 10% ammonium acetate : methanol (1:1) system, and this allows the unreacted gallium to remain at the origin on the cellulose system but moves the unreacted indium to the same R_f as the chelated form. As stated before, systems that gave R_f values less than 0.5 for the chelate compound did not give well defined separation of the unreacted radiometal and chelated radiometal. A good example of this is ^{111}In-PAB-EDTA and ^{111}InCl$_3$ on the cellulose system developed in 70% acetonitrile, pH=2.0. The PEI cellulose system developed here is the only one that can be used to separate both unreacted ^{111}In and ^{67}Ga from their chelated forms.

As well, these systems can only be applied to highly acidic solutions of the radiometals and PAB-EDTA. Experiments using buffers such as citrate or acetate show that unreacted radiometal no longer remains at or near the origin and moves with R_f values similar to that of the PAB-EDTA in the cellulose and PEI cellulose systems. Of course, the exception to this is the silica gel system where even with the presence of weakly chelating buffers, ^{67}Ga does not move with an R_f greater than 0.1 but ^{111}In can be resolved into unreacted ^{111}In and chelated ^{111}In.

EDTA complexed with ^{67}Ga and radioiodinated PAB-EDTA were also tested on three of the systems for comparison. The EDTA complex had R_f values of 0.7 to 0.95. This increased R_f could be related to the difference in structure between PAB-EDTA and EDTA. The lack of the derivatized benzyl ring might allow the complex to partition more easily into the solvent and move farther up the stationary phase. The radioiodinated compound had virtually the same R_f value as the radiometal chelated compound

indicating that the presence of the radioiodine molecule on the benzyl ring did not significantly alter the chromatographic properties of the compound.

3.1.3.2 CHELATE STABILITY

The stability of ^{111}In-PAB-EDTA in the presence of apo-transferrin and HSA was tested over a 72 hour period. ^{111}In, when injected I.V. as ^{111}In-chloride, is known to bind very rapidly to transferrin. It is important therefore to determine the stability of the chelate complex in the presence of transferrin if the chelate is going to be applied to *in vivo* diagnostic use. The results of the stability study are summarized in Table 6 and indicate that the chelate complex is very stable in the presence of transferrin. The ^{111}In-citrate was tested with albumin as a control for non-specific binding of the chelate complex to albumin. Values less than 100% can be attributed to ^{111}In loss from the chelate or non-specific adsorption of the chelate to transferrin or albumin. In view of the high stability in the presence of transferrin, it is more likely that the small changes seen with the complex in the presence of albumin are due to non-specific adsorption of 1 to 2% of the chelate.

3.1.4 ANALYSIS OF RADIOMETALS

3.1.4.1 THIN LAYER CHROMATOGRAPHY

Initial testing of the radioisotope solutions used for chelation studies was performed on TLC. For $^{111}InCl_3$, an R_f of 0.0 on silica gel in either 10% ammonium acetate : methanol (1:1) or acetone, pH=2, should be obtained if no chelate contaminants are present.[146] When injection grade grade $^{111}InCl_3$ (Medi-Physics) was chromatographed on these systems and on PEI cellulose in 1 M LiCl, pH=1.0, 98% of the radioactivity moved with R_f values of greater than 0.75, 0.40 and 0.70, respectively, indicating the presence of contaminants. Chromatography of reaction grade $^{111}InCl_3$ (AECL) showed R_f values of 0.1 and 0.0, respectively, for the silica gel systems indicating no contamination present. Injectable grade ^{67}Ga-citrate (Frosst) showed the

TABLE 6. Stability of ^{111}In-PAB-EDTA in the Presence of Apotransferrin and HSA.

Complex/Protein	Percent of the Activity Retained on the Complex* after Incubation with the Protein** for the Following Times		
	24 hr	48 hr	72 hr
^{111}In-PAB-EDTA/Tf	99.6	99.7	99.6
^{111}In-PAB-EDTA/HSA		97.1	96.4
^{111}In-citrate/Tf	0.30	0.27	0.32
^{111}In-citrate/HSA	98.8		99.7

* analysis by gel chromatography;
** incubated at 37°C

expected R_f values of 0.7 and 0.5 when chromatographed on cellulose in 10% ammonium acetate : methanol (1:1) and acetone, pH = 2.0 and reaction grade $^{67}GaCl_3$ (Frosst) showed R_f values of 0.1 and 0.2, respectively, in the same system. The ^{67}Ga-citrate preparation was expected to produce R_f values that corresponded to the chelate R_f values due to the presence of citrate. Contaminants in the injectable $^{111}InCl_3$ were not identified but presented problems when trying to label chelated proteins. Although the $^{67}GaCl_3$ chromatographed as a pure compound, i.e. it did not have chelate contaminants, the presence of non-radioactive metal ions that could not be easily detected by TLC produced interference and reduced labeling yields with chelated proteins.

Figure 4 summarizes the effects of two non-radioactive metal ions, iron (Fe) and zinc (Zn), on the chelation of $^{111}InCl_3$ and $^{67}GaCl_3$ to PAB-EDTA in 0.1 N HCl. The presence of non-radioactive metals in the radioisotope solutions was first suspected when problems were encountered when trying to label the "no-carrier-added" $^{67}GaCl_3$ to small amounts of PAB-EDTA. Further evidence was obtained from the supplier that indicated the possible presence of significant amounts of copper (Cu), titanium (Ti), Zn and Fe, which could possibly compete with the ^{67}Ga for chelation with PAB-EDTA. Based on the instrumental neutron activation analysis (INAA) (see below), Zn and Fe were used in various molar ratios to determine their effect on the chelation of both $^{111}InCl_3$ and $^{67}GaCl_3$ to PAB-EDTA. The ratios of Zn or Fe : PAB-EDTA ranged from 0.1:1 to 100:1. As seen in Figure 4, $FeCl_3$ dramatically reduces the chelation of both radioisotopes to PAB-EDTA with increasing $FeCl_3$: PAB-EDTA ratios. This is not unexpected, since iron has a slightly higher binding constant for underivatized EDTA than indium or gallium.[162] At a ratio of 1:1, $FeCl_3$ has almost completely eliminated the binding of both radioisotopes, indicating that PAB-EDTA also exhibits the same preferential chelation of Fe over gallium or indium as underivatized EDTA. $ZnCl_2$ requires higher ratios to produce interference with radiometal chelation and has more effect on ^{67}Ga than on ^{111}In. At ratios of 100:1 Zn : PAB-EDTA, 50% of the ^{67}Ga is displaced whereas only 30% of the ^{111}In is displaced. At ratios of as little as 5:1 Zn : PAB-EDTA, the amount of

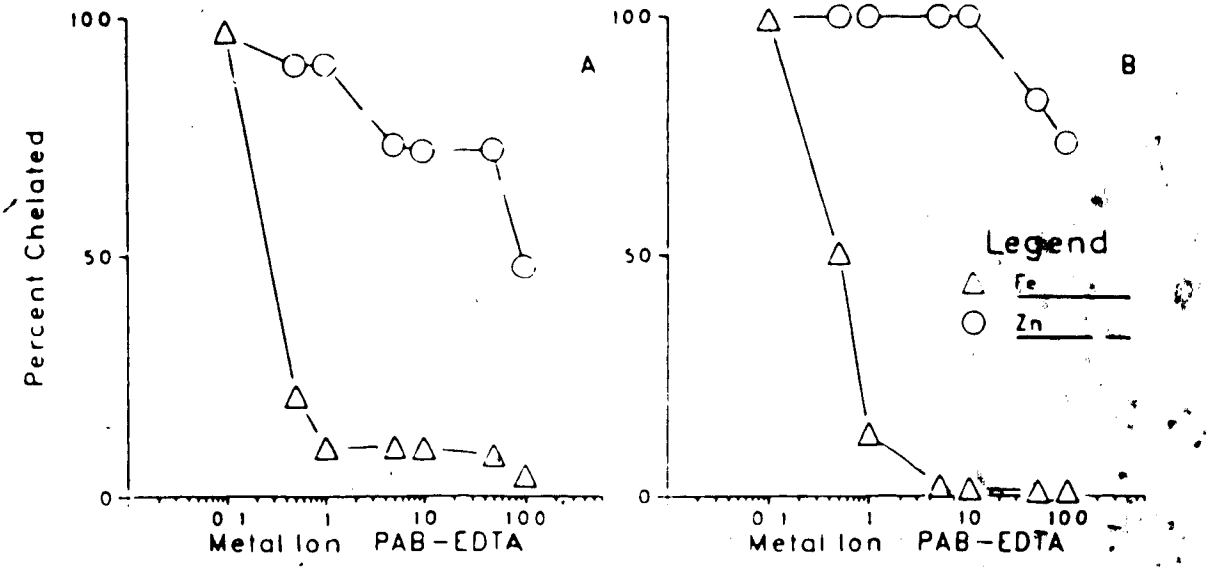


Figure 4. Effect of iron (Fe) and zinc (Zn) on the chelation of A) ^{67}Ga and B) ^{111}In by PAB-EDTA in 0.1 N HCl.

displaced ^{67}Ga is 30% with the ^{111}In unaffected. Any Zn already present in the radiogallium preparation would not be having an additive effect as both radioisotope solutions were diluted 1 in 100 before use in this study.

3.1.4.2 INSTRUMENTAL NEUTRON ACTIVATION ANALYSIS

Instrumental neutron activation analysis (INAA) is a technique that is suitable for multi elemental, non-destructive analysis and is applicable to small sample volumes. The radioisotope solution, once decayed, is easily assessed by this technique which involves the bombardment of the sample with thermal neutrons in a reactor. Specific elements present in a sample will capture thermal neutrons (neutron activation) and become radioactive. The detection of these radioactive elements using sensitive detectors and the comparison to standard amounts of the elements activated under identical conditions, allows both identification and quantification of these elements.

The irradiation schedule chosen was based on:

1. an estimate of the elements that may be present in the solutions prior to irradiation,
2. selection of the appropriate radioisotope produced from these elements by neutron activation and
3. the effect of background radioisotopes that are generated by other elements in the sample.

For example, sodium and chlorine are commonly found in both radiogallium and radioindium preparations, they can be easily activated, can contribute significantly to the background and can interfere with the detection of the radioisotopes of interest. Table 7 summarizes the relevant INAA data for detection of the elements used in this investigation.[22]

To reduce the effect of sodium and chlorine, two irradiations were chosen. A short irradiation of 5 minutes at maximum flux was used to activate the short half-life radioisotopes, Cu and Ti. A long irradiation of 4 hours at maximum flux was used to activate the longer lived isotopes, Zn, Fe and Cd. For the long irradiation, a 96 hour cool period for Cd and a two week cool period for Zn and Fe, was necessary before analysis to

TABLE 7. Neutron Activation Data for Selected Elements

Element	Relevant Isotope	Half-life	Major γ-Ray* Energy in keV (%)	Calculated** Detection Limit (μg/sample)
Cu	^{66}Cu	5.10 min	1039 (8)	1.0
Ti	^{51}Ti	5.80 min	320 (93)	2.0
Zn	^{65}Zn	244.1 days	1116 (51)	0.5
Fe	^{59}Fe	44.6 days	1099 (56)	10
Cd	^{115}Cd	58.4 min	336 (50)	2.0
			528 (34)	
			492 (11)	
Na	^{24}Na	15.0 hours	1369, 2754 (100)	--
Cl	^{38}Cl	37.3 min	2168 (42)	--

* From 'Table of Isotopes, 7th ed.' Lederer CM and Shirley VS, eds. John Wiley and Sons, Toronto. 1978.

** From Currie LA, Quantitative determination - application to radiochemistry. *Anal. Chem.* 40:586 1968.

allow the activated sodium and chlorine to decay to acceptable levels. Detection limits (summarized in Table 7), based on irradiated standard and background activities, were calculated according to the method of Currie[156] and are necessary to estimate the minimum amount of an element which can be determined in a sample under the conditions chosen. Initial counting was performed on the Ge(Li) spectrometer system to assess the elements present.

Cu, Ti and Cd were not detected in any of the radioisotope samples irradiated. Trace amounts of Fe were detected and up to 1.27 mg/ml of Zn were found in the radiogallium samples assayed. Zn and Fe were not detected in the radioindium samples analyzed. Figure 5 shows the Ge(Li) spectrum of a 100 μg Zn and Fe standard compared with a typical sample of decayed $^{67}GaCl_3$.

Samples from the long irradiation were also counted in a NaI(Tl) multi sample well counter. Although the Ge(Li) system has a higher resolution than the NaI(Tl) system, the latter was used to quantitate more accurately the amount of Zn and Fe present in the radiogallium samples due to its higher counting efficiency. Table 8 summarizes the elements found in the various batches of radiogallium and radioindium.

The analysis of these samples for long lived isotopes that result from neutron activation present a special problem. Suitable radioactive elements are not always available due to the inherent characteristics of these elements such as small neutron cross sections and very long half lives. These drawbacks are outweighed in this case by the ease of multi element analysis of the samples as compared to a technique such as atomic absorption and provide an elegant method to quantitate the metal ion impurities in these samples.

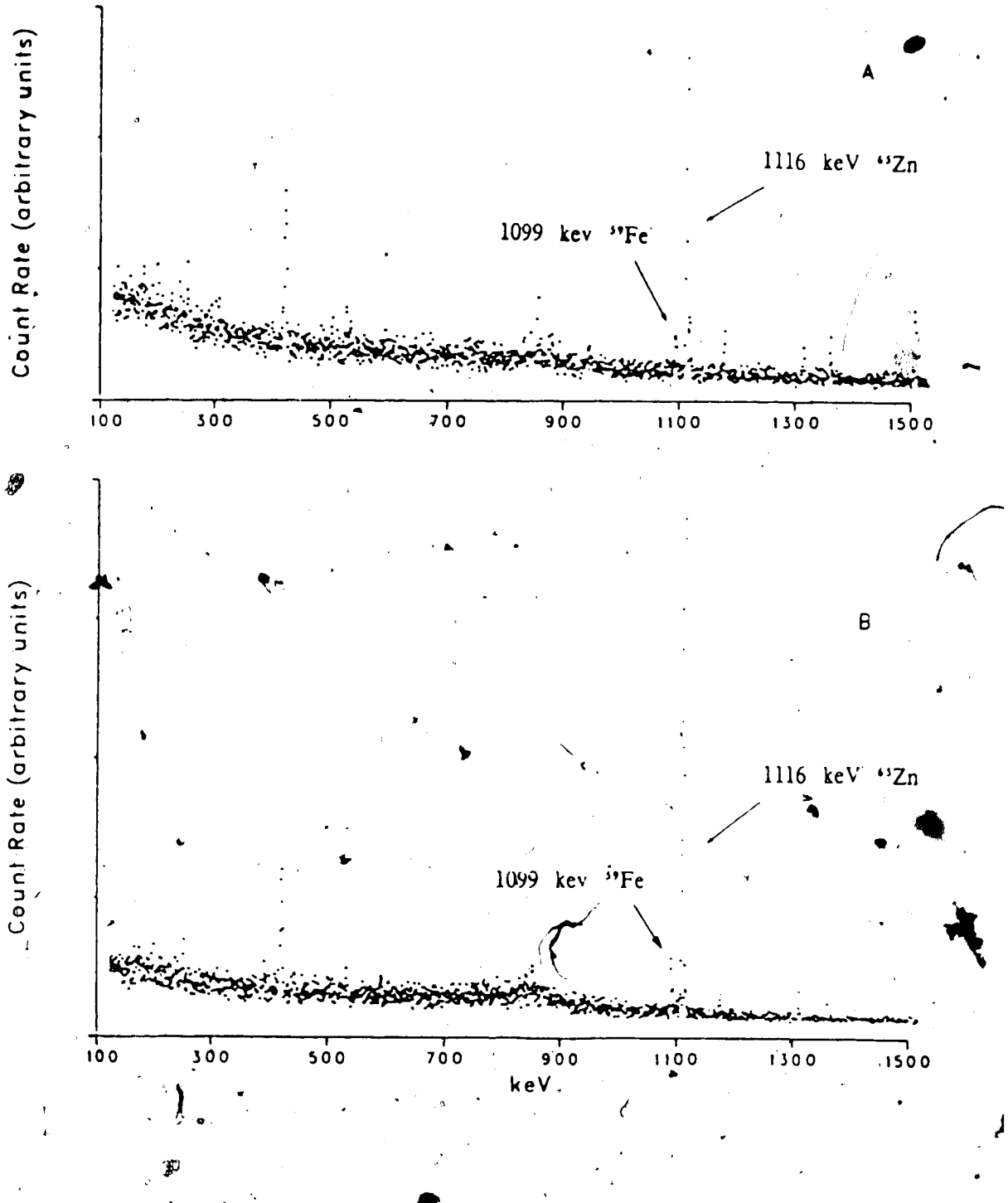


Figure 5. Ge(Li) spectra of A) a typical decayed $^{69}GaCl_3$ sample and B) 100 μg iron and zinc standards 2 weeks after thermal neutron irradiation for 4 hours at 10^{12} n·cm^{-2}·sec^{-1}.

TABLE 8. Neutron Activation Analysis of Decayed Radiogallium and Radioindium Chloride

Radiogallium[1]			Radioindium[1]		
Batch	Zn[2]	Fe	Batch	Zn[2]	Fe
A	0.66	N.D.[3]	A	N.D.	N.D.
B	0.25	trace[4]	B	N.D.	N.D.
C	1.27	N.D.	C	N.D.	N.D.
D	0.23	N.D.	D	N.D.	N.D.
E	0.40	N.D.	E	N.D.	N.D.
F	0.54	trace	F	N.D.	N.D.
G	0.92	N.D.	G	N.D.	N.D.

[1] Cu and Ti were not detected - <1.0 and <2.0 μg/sample respectively in radiogallium; Cd not detected - <2.0 μg/sample in radioind[illegible]
[2] mg/ml
[3] N.D. = not detected - <10 μg/sample for Fe; <0.5 μg/sample for Zn
[4] trace = 10-20 μg/sample

3.1.5 PURIFICATION OF RADIOMETALS

3.1.5.1 ION EXCHANGE CHROMATOGRAPHY

Ion exchange chromatography and, in particular, anion exchange chromatography has been used successfully for purification of both ^{111}In and ^{67}Ga. The procedure is based on the premise that in very high concentrations of HCl (2 to 3 N or greater) the neutral trichloride salt of indium or gallium becomes negatively charged due to the association of a fourth chloride ion.[163] This allows the binding of the negatively charged tetrachloride species to the anion exchange media, in this case AG 1 x 8 (BioRad), that is also equilibrated with a high concentration of HCl. The binding of the the tetrachloride species depends greatly upon the molarity of the HCl used[164] and the chloride complexes of Zn, Fe, In and Ga can be separated due to their differential binding under the conditions used. This method has been used to separate radioisotopes produced by spallation reactions on an industrial scale[165] and is the basis for many radioisotope generator systems.[166,167] Since Zn and Fe seem to be the major contaminants in ^{67}Ga and to a smaller degree in ^{111}In this method should produce high purity ^{111}In and ^{67}Ga. Care must be taken not to re-introduce contamination with the eluting HCl and ultrapure HCl (BDH) was used for this purpose.

The purified ^{111}In and ^{67}Ga is usually eluted in dilute form but can easily be concentrated by evaporating the 0.1 N HCl and storing the isotope dry until needed. Redissolution in a small quantity of 0.1 N HCl is then easily acccomplished and the isotope is ready for use.

The results with ^{111}In, shown in Table 9, indicate that the 2 N HCl used by Leung[161] and Meares[51] does not adequately retain the $InCl_4^-$ complex. The maximum binding of indium should occur in 4 N HCl[164] and the best results were obtained when this molarity of HCl was used. Labeling efficiencies of up to 60% were obtained with the purified $^{111}InCl_3$ on chelate labeled MAbs indicating that the purification process had improved the chelation of the ^{111}In since the unpurified $^{111}InCl_3$ (injection grade) could not give labeling efficiencies greater than 5%. Purification results were not available for

TABLE 9. Purification of $^{111}InCl_3$* By Anion Exchange.

Conc. of HCl for ^{111}In Binding to Column	Percent Recovered By 0.1 N HCl Elution	Radiochemical Purity** of stock	Radiochemical Purity** after elution
2.0 M	50	90	100
4.0 M	99	83	100
6.0 M	84	83	100

* obtained from Medi-Physics Inc.
** analysed by silica gel TLC with 10% ammonium acetete:methanol (1:1) solvent

$^{67}GaCl_3$, but based on the fact that zinc is the major contaminant in these solutions and that zinc has a different binding molarity than gallium, this method would appear to be an excellent way to purify the $^{67}GaCl_3$ solution prior to use with chelated proteins.

3.1.5.2 ETHER EXTRACTION OF RADIOGALLIUM UNDER REDUCING CONDITIONS

This technique is used specifically for gallium and in particular for ^{67}Ga obtained from generators either as the EDTA complex or in ionic form in 1 N HCl. The use of a reducing agent ensures that metallic impurities such as Fe and Zn are maintained in the +2 state which is non-ether extractable. According to Maziere,[154] silver metal produced the best results due to its virtual insolubility in hydrochloric acid solutions. They reported less than 5 x 10^{-6}% of the metallic reducing agent (<10 ng of Ag) and approximately 4 ng of Fe and 6 ng of Sn were present in a 7 mCi (260 MBq) batch of ^{67}Ga eluted from a SnO_2/HCl ionic ^{67}Ga generator. When $^{67}GaCl_3$ (Frosst) was purified by this method, TLC of the purified $^{67}GaCl_3$ on cellulose in 10% ammonium acetate : methanol (1:1) showed only one spot near the origin. However, when used in labeling chelated proteins, the results were not satisfactory as still less than 20% of the ^{67}Ga was chelated.

The TLC method of analysis only shows that there are no [illegible] impurities present and does not show non-radioactive metal ion contamination. Again, strict control of non-radioactive metal ion contamination is important and with this method, not as easily controlled. Metal ions of the +3 valence in the chloride form are easily extracted into the ether solution and may already be present in the ether used. Further analysis was not attempted to determine the impurities that may have been present. The ion exchange method for purifying $^{67}GaCl_3$ may yield better results.

3.1.6 PURIFICATION OF MAb-155H.7

The use of Protein A coupled to a solid support is a standard method for the purification of IgG-type antibodies.[169] Protein A, a bacterial lectin consisting of a single polypeptide chain with an approximate molecular weight of 42,000 and usually isolated from a

strain of *S. aureus* that does not incorporate the protein into its cell wall, has been shown to bind to the Fc portion of IgG molecules and very weakly, if at all, to IgA, IgM or IgE antibodies of many mammalian species.[170] As well, certain subclass specificity has been shown and Protein A will bind strongly to murine IgG of subclass 2a, 2b and 3 but weakly to subclass 1. IgG's of subclass 1 can be purified on Protein A but this requires the use of a "Binding Buffer" commercially available from BioRad. The exact ingredients of this buffer are unknown but the results can be remarkable. It has also been reported that separation of subclasses of murine IgG can be effected using immobilized Protein A.[171]

MAb-155H.7 was determined by initial and subsequent screening using the Mouse Typer kit (BioRad) to be of the IgG_{2b} subclass which made purification by the Protein A method a logical choice. Other methods can be used successfully for the purification of IgG antibodies, such as DEAE-cellulose (DE52 - Whatman) or HPLC but Protein A was chosen for the ease in handling and manipulation offered by this particular gel.

The ascites fluid was clarified by centrifugation prior to the separation to remove particulate matter and lipids. These materials, if left in the ascites preparation, will obstruct column flow and impede the elution process. The clarified ascites could then be diluted 1:1 with starting buffer or ammonium sulfate precipitated. The latter procedure was employed when very large amounts of ascites required purification. Ammonium sulfate not only concentrates the protein of interest it also removes a large portion of the unwanted, non-specific proteins such as albumin from the preparation. Thirty-three percent ammonium sulfate should precipitate essentially all the immunoglobulins and eliminate approximately 75% of the irrelevant proteins in the ascites fluid.[172] The most soluble protein, albumin, only completely precipitates in the presence of 100% saturated ammonium sulfate. In this case two precipitations were performed to ensure a high recovery of MAb-155H.7. Initial screening of the supernatant and redissolved 33% ammonium sulphate precipitate indicated that a substantial amount (30%) of MAb-155H.7 remained unprecipitated. Therefore, a 50% ammonium sulphate precipitation followed by a 33% precipitation was adopted to produce high recoveries of the MAb of interest.

Salting out of proteins in this manner is an essentially mild form of precipitation and causes little protein denaturation. The major drawback is the time consuming dialysis needed to remove the large amounts of salt still remaining in the redissolved precipitate.

The elution of the antibody from the column was performed by a step-wise gradient of 0.1 M sodium citrate from pH=5.5 to 3.0. Each fraction collected was screened by ELISA for MAb-155H.7 activity to Tβ and by protein assay (BioRad) for protein concentration. ELISA of the ascites before and after purification indicated virtually no loss of *in vitro* antigen binding activity.

Table 10 summarizes the *in vitro* binding activity of the various peaks collected from the Protein A column expressed in terms of the amount of protein present equated to absorption units at 405 nm in the standard ELISA described previously. This index was used to obtain a standardized method of comparing different batches of purified MAb and to compare the effects of subsequent manipulations.

Although the peak at pH=3.0 contains similar activity to that obtained at pH=4.0, the total amount of protein suggests that this is a small fraction of the active antibody that is retained by Protein A and only elutes at pH=3.0. Ey *et al.*[171] have suggested that IgG_1 antibodies elute between pH 5 and 6, IgG_{2a} antibodies elute betweeen pH 4 and 5 and that IgG_{2b} antibodies elute between pH 3 and 4. Since this antibody has been well characterized as an IgG_{2b}, it would appear not to follow this pattern. Stephensen *et al.*[173] described the purification of a large number of murine monoclonals of IgG subclasses and showed that not one of the IgG_{2b} antibodies purified completely followed the scheme described by Ey. Although there seems to be a relationship to the pH used and antibody subclass, a generalization cannot be applied to all antibodies.

The screening of purified MAb-155H.7 was performed not only on the hapten-protein complex used for immunization but also on other conjugate forms of the Tβ-hapten and native HSA. Table 11 compares these results with those obtained when the immunizing complex is used in the standard ELISA.

TABLE 10. Analysis of Protein A Purified MAb-155H.7 From Mouse Ascites.

Protein A Elution pH	Ascites from 0-3 Month Old Clones		Acites from 3-6 Month Old Clones	
	pg/A_{405}*	mg/ml Ascites	pg/A_{405}*	mg/ml Ascites
5.5	35324	0.25	31300	0.01
4.0	82	3.16	2024	0.49
3.0	206	0.06	1162	0.01

* see text, pg 73
Note: each value is the mean of 3-6 determinations, s.d. < 15%

TABLE 11. Binding of MAb-155H.7 to Various Tβ Conjugates by ELISA.

ELISA units	Tβ-HSA (Batch 1)	Tβ-HSA (Batch 2)	Tβ-KLH	Tβ-BSA	HSA
pg/A_{405}* (1)**	82				270
(2)**	2024	16483	0.0	0.0	10390

* see text, pg 73
** (1) Ascites from 0-3 month old clones; (2) Ascites from 3-6 month old clones
Note: each value is the mean of 3-6 determinations, s.d. < 15%

The non-reactivity of MAb-155H.7 to the KLH and BSA conjugated forms of the Tβ-hapten is unexpected and the reduced binding to Tβ-HSA of a lower hapten : protein ratio is also surprising. The binding to HSA is relatively strong compared to the immunizing conjugate and would suggest that MAb-155H.7 contains as part of its antigen binding site a region that strongly recognizes HSA.

The reasons for the variability of binding by MAb-155H.7 to the various synthetic haptens could be explained in a number of ways. One reason could be that the configuration of the Tβ-hapten on the various protein carriers as compared to the immunizing complex does not sufficiently mimic the structure of the original, highly conjugated complex and therefore does not 'fit' the antigen binding site of MAb-155H.7. The relatively high affinity of MAb-155H.7 for HSA could also play a role in the reduced binding seen to the BSA and KLH conjugates. [redacted] is important for antigen recognition, the structures of BSA and KLH could be sufficiently different to cause the MAb to not recognize the Tβ-hapten and not bind specifically to these conjugates. MAb-155H.7 does not cross react with BSA and this is demonstrated by the fact that addition of BSA to the diluting buffer does not inhibit the binding of MAb-155H.7 in the standard ELISA. This data was supported by a lack of differential binding between diluting the MAb in 1% BSA / PBS and in PBS alone. These studies suggest that the binding of MAb-155H.7 to the synthetic antigens is more complex than just recognizing the Tβ-hapten on the conjugate and that the MAb requires the presence of HSA as the carrier protein for the hapten to bind specifically to the synthetic hapten-conjugate.

3.1.7 LABELING TECHNIQUES

3.1.7.1 METHODS OF ANALYSIS

GEL FILTRATION

The separation of labeled protein from unreacted chelate or unreacted radiometal or radioiodine using gel exclusion chromatography is based on molecular weight size differences. The three gels tested had a range of molecular weight exclusion limits that could effectively separate the labeled protein from the unreacted products. BioGel P-100 (BioRad) has a fractionation range of 5,000 to 100,000 a.m.u. Sephadex G-50 fine (Pharmacia) has a range of 1,500 to 30,000 a.m.u. and BioGel P-6DG (BioRad), a desalting gel, has a range of 1,000 to 6,000 a.m.u.

The standard method of purifying radiolabeled protein by conventional gel filtration was compared with a centrifuged column gel filtration technique. The conventional method is relatively simple and prpduces high yields of recovered protein and is highly reproducible.[174,175] The major drawback is the time required to complete the separation with a typical fractionation run using gels that fractionate in the 150,000 dalton range or greater being anywhere from 1 hour to several days. The desalting gels are considerably faster due to their structure and separations on these gels can be completed in as little as 30 minutes. Another disadvantage is the considerable sample dilution that takes place especially in fractionation runs. For radiolabeled proteins of low specific activity, this could be a major problem if subsequent use requires a high specific activity in small volumes (i.e. biodistribution studies and cell uptake studies). As well, accessory equipment such as flow through UV monitors and fraction collectors [illegible] necessary and expensive.

Many of these concerns can be eliminated or vastly simplified if the centrifuged column technique is employed. This technique is also simple, produces good protein recoveries and is highly reproducible. The major advantages are the

saving in time as it reduces a separation to less than 15 minutes as compared to hours for conventional gel filtration. The recovered sample is highly concentrated. Accessory equipment is usually available to a table top, centrifuge and the column apparatus can be readily manufactured from available laboratory equipment.

In the experiments described here, conventional gel filtration was used to establish baseline parameters and then the centrifuged column technique was adapted to these conditions. The centrifuged column technique was assessed for protein recovery, unlabeled chelate breakthrough and unlabeled radioiodine and radiometal breakthrough under various conditions. Table 12 summarizes this data.

The best conditions for maximum protein recovery utilize the table top centrifuge with a maximum speed of 100 x g for 2 minutes with subsequent washes after the initial centrifugation. Protein assay using the BioRad kit was used in one experiment and supports the data generated by ^{125}I-IgG. The 80 to 85% protein recovery reported here is somewhat lower than that reported by Penefsky[155] (98 to 100%), for a similar centrifuged column system using Sephadex G-50 fine as the gel material but correlates well with the percent protein recovery reported by Saul and Don[176] and Meares *et al.*[31] for similar centrifuged column systems using Sephadex G-25 (Pharmacia) and Sephadex G-50-80 (Sigma) respectively.

In the case of BioGel P-100, protein recovery is the sum of the first 3 fractions collected from the column and for Sephadex G-50 fine is the sum of the first 2 fractions. The addition of a 100 µl wash after the initial centrifugation improved protein recovery in all cases. In the case of no wash being added, further protein is not recovered due to loss of buffer in the gel beads. If buffer is added, the gel is rehydrated and the trapped protein can be recovered. According to Penefsky,[155] after the initial pre-centrifugation before sample addition, the gel beads are dehydrated considerably especially in the top one-third of the column. With addition of the protein sample, the gel absorbs the buffer in the sample along with most unbound ions and causes the protein to dehydrate. The protein can then be recovered

TABLE 12. Percent Protein Recovery from Spun Mini-columns.

Centrifuge Type	Spin Time in min. RPM (x g)	Column Gel	Recovery of ^{125}I-IgG with wash*	without wash*
Dynac table top	15 2755 (1240)	BioGel P-100	51.1†	59.0±10.0
	5 2755 (1240)	BioGel P-100		77.1±5.3
	2 830 (140)	BioGel P-6DG	45.0±0.8	29.0†
		Sephadex G-50	0.0	1.2†
	2 670 (100)	BioGel P-100	76.8±6.0 79.1±4.4**	
		BioGel P-6DG	72.3±2.4	
		Sephadex G-50	85.6±3.5	
IEC floor model	2 500 (45)	BioGel P-6DG	22.2±1.8	0.0
		Sephadex G-50	0.0	0.0
	2 750 (100)	BioGel P-100	53.0±9.5	35.7±3.8
	5 750 (100)	BioGel P-100	31.9±4.4	0.9±0.2
		Sephadex G-50	0.0	0.4±0.1
	5 1000 (180)	BioGel P-100	69.8±7.5	68.6±4.8
	5 2000 (720)	BioGel P-100		73.3±1.6

* Wash = 100 µl of buffer added to column after sample centrifugation followed by a second spin
** Determined by protein assay
† Single determination, all others x ± s.d. of 3 to 7 values

in a highly concentrated form in a volume of buffer that is held within the lower portion of the column. For poly-H-IgG, the best conditions turned out to be similar to those selected by Meares *et al.*[11] and it was found that increasing the g-force or changing the centrifuge had significant effects on protein recovery.

The choice of gel system to use was based not only on protein recovery but also on radioiodine, radiometal and chelate breakthrough in the presence of protein in these various systems. These parameters are summarized in Table 13:

Although breakthrough of unwanted compounds is less when no wash is applied to the column, the protein recovery is less (see Table 12). Chelate, radiometal and radioiodine breakthrough were all less than 1.5% and this small amount of breakthrough could be attributed to non-specific adsorption to the protein used. When these columns were tested in the absence of protein, chelate, radiometal and radioiodine breakthrough was consistently less than 0.5%.

Protein recovery on the gels tested is virtually the same in all cases ranging from 70 to 80%, with BioGel P-100 and Sephadex G-50 fine slightly better. Chelate, radiometal and radioiodine breakthrough was reduced on BioGel P-100 as compared to Sephadex G-50 fine and this could be a function of the exclusion size of gel. When compared in conventional gel chromatography, retention times for small molecular weight molecules is less on Sephadex G-50 fine than BioGel P-100 and the Sephadex gel has a smaller fractionation range than BioGel P-100. Total protein recovered on the BioGel P-100 system was achieved only after two additional washes of the mini-column whereas on Sephadex G-50 fine, total protein was recovered in the first centrifugation after sample application. In some cases, an additional wash had to be used to achieve the desired result. This can also be explained by the fractionation range with the BioGel P-100 exclusion limit being closer to the molecular weight of IgG than Sephadex G-50 fine. In spite of the fact that total protein recovery on BioGel P-100 required two washes after sample application, less contaminant breakthrough made this the system of choice.

TABLE 13. Radiometal and Radioiodine Breakthrough on Spun Mini-columns.

Centrifuge Type	Spin Time in minutes RPM (x g)	Column Gel	^{67}Ga-B-EDTA with wash*	^{67}Ga-B-EDTA without wash*	^{67}GaCl$_3$ with wash*	^{123}I with wash*
Dynac table top	15 2755 (1240)	BioGel P-100	0.77†			
	5 2755 (1240)	BioGel P-100				7.79†
	2 670 (100)	BioGel P-100	1.04±0.01		1.22±0.05	0.18†
		Sephadex G-50	1.21±0.06		1.48±0.27	1.00±0.28
IEC floor model	2 750 (100)	BioGel P-100	1.06±0.74	1.18±0.63		
	5 1000 (180)	BioGel P-100	1.34±0.67	0.62±0.04		
	5 2000 (740)	BioGel P-100		0.82±0.2		

*Wash = 100 µl of buffer added to column after sample centrifugation followed by a second spin
† Single determination, all others x ± s.d. of 3 to 5 determinations

THIN LAYER CHROMATOGRAPHY

The TLC systems used to assess reaction mixtures for efficiency of chelate labeling, rely on separating protein labeled chelate groups from unlabeled chelate groups. For ^{111}In, the silica gel and 10% ammonium acetate : methanol (1:1) system keep the protein at the origin (R_f = 0.0) and move unlabeled chelate to an R_f of 0.8. For ^{67}Ga, the cellulose and 10% ammonium acetate : methanol (1:1) system produces results similar to those of the silica gel system with protein remaining near the origin and the unlabeled chelate moving with an R_f of ~0.75. Both of these systems must have an excess of chelate present to move non-protein bound ^{67}Ga or ^{111}In from the origin and cannot be used to analyze purified protein unless an 'EDTA challenge' is first made to scavenge any unlabeled radiometal. This method is not satisfactory since a minor miscalculation can add excessive EDTA to the protein mixture and turn the equilibrium in favour of EDTA thereby removing bound radiometal from the labeled protein. To overcome this, a third system was developed that could move unlabeled ^{111}In and ^{67}Ga from the origin in the presence or absence of free chelate. The results of the cellulose with methanol : 0.1 N HCl (7:3) solvent are presented in Table 14. In this system only chelated radiometal remained at the origin.

The use of standardized $^{111}InCl_3$ to assess the number of chelate groups labeled to protein is similar to the $^{57}CoCl_3$ assay developed by Meares *et al.*[51] The major drawback is that this is only an estimate and does not give an exact chelate : protein ratio. The assay depends upon the accurate preparation of the standardized solutions and is hampered by contaminating metal ions such as Fe and Zn that can compete with the radiometal for chelation to the protein bound chelate groups. Meares *et al.*[51] have suggested that the minimum concentration of chelating groups bound to protein that can be accurately assessed is 10 μM. Anything less than this is reportedly below the background concentration of contaminating metal ions. This limit was calculated based on the precautions needed to avoid extraneous metal ion

TABLE 14. TLC* R_f Values for Radiometals and Radiochelated poly-H-IgG

Compound	R_f	Compound	R_f
$^{67}GaCl_3$	0.75	$^{111}InCl_3$	0.71
^{67}Ga-citrate	0.75	^{111}In-citrate	0.71
^{67}Ga-poly-H-IgG	0.15	^{111}In-poly-H-IgG	0.20

*TLC system used: Methanol : 0.1N HCl (7 : 3) on Cellulose

contamination such as acid washing glassware and treating buffers to reduce this background. In these studies, when concentrations of purified protein that contained less than 10 μM chelate were re-assessed by the standard TLC for number of chelate groups present, consistent results were never obtained and always indicated a chelate ratio far less the first estimate from the reaction mixture. When the concentration was above 10 μM, consistent results were always obtained.

More accurate measurements can be obtained if a radioactive label such as ^{14}C or ^{3}H is incorporated directly into the chelating agent as it is synthesized. This technique was not available for these studies and repeated analysis by the TLC systems or conventional gel chromatography had to be relied upon to determine the labeling efficiency of the chelate reactions.

The effect of non-radioactive contaminants on the chelation of ^{67}Ga and ^{111}In to chelate labeled proteins was also assessed using TLC on cellulose with methanol : 0.1 N HCL (7:3) as the solvent as described above. From the INAA experiments, it was determined that the significant amount of $ZnCl_2$ present in the $^{67}GaCl_3$ preparation had deleterious effects on the chelation of ^{67}Ga to unconjugated chelate under acidic conditions. The attempt was made to study these effects using chelate labeled poly-H-IgG as a model and the radiometals ^{111}In and ^{67}Ga. The concentration of chelate was kept above the 10 μM limit and was performed in 0.1 M citrate buffer at pH=6.0 to keep ^{67}Ga and ^{111}In soluble at the more neutral pH. The results are presented in Table 15 and indicate there are significant problems in trying to chelate ^{67}Ga to the labeled protein. When the interference study with Zn and Fe was attempted with the ^{67}Ga-B-EDTA-poly-H-IgG system, the baseline chelation was 2% and any added Zn or Fe reduced this completely to 0. The effect of added Zn and Fe was successfully studied using ^{111}In and these results are shown in Figure 6. In this case, Fe-citrate had a similar effect to that of $FeCl_3$ in reducing the amount of ^{111}In chelated. Surprisingly enough, zinc-citrate had a significantly higher effect on the chelation of ^{111}In by PAB-EDTA than $ZnCl_2$. When Zn- and Fe-citrate

TABLE 15. Binding of ^{67}Ga and ^{111}In to B-EDTA-poly-H-IgG

Amount of Chelate on Protein (nmoles)	% of Radioisotope Bound*	
	^{67}Ga	^{111}In
0.066	0	92
0.663	4.1	93
6.63	6.6	98
66.3	8.9	98
663	13.3	98

* 1 μl of stock radioisotope solution

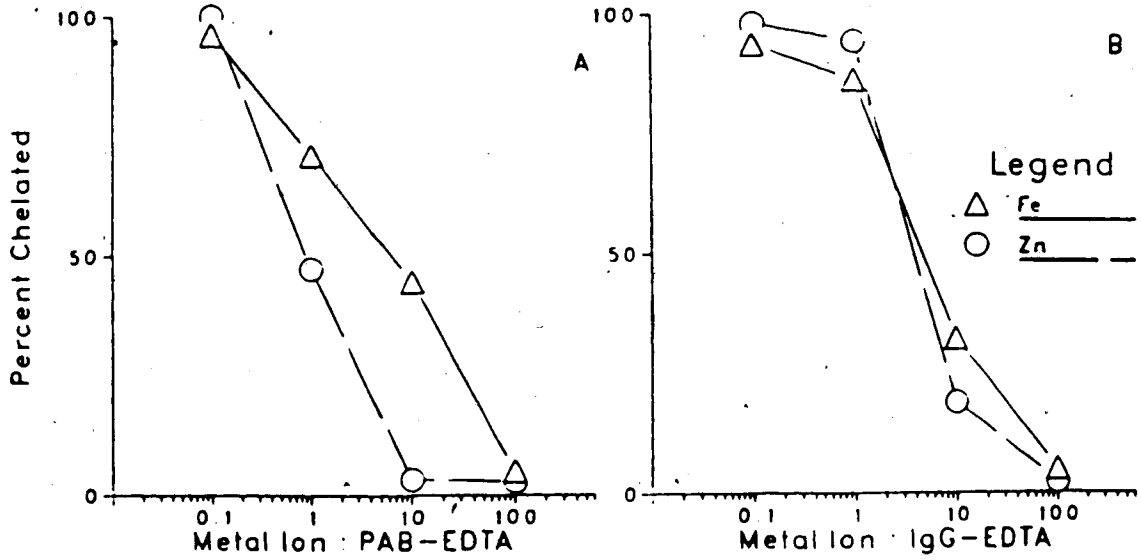


Figure 6. Effect of iron (Fe) and zinc (Zn) on the chelation of ^{111}In by A) PAB-EDTA and B) B-EDTA-poly-H-IgG in 0.1 M sodium citrate, pH = 6.

interference studies were conducted with B-EDTA-poly-H-IgG, both metals followed a similar pattern and reduced the binding of ^{111}In to less than 40% at a ratio of 10:1 metal ion : B-EDTA-poly-H-IgG. This could explain the problems encountered with ^{67}Ga chelation from ^{67}Ga-citrate to B-EDTA-poly-H-IgG. The amount of Zn already present in the undiluted stock when transformed into Zn-citrate would be enough to prevent the chelation of ^{67}Ga to the labeled protein. The average amount of Zn present in the $^{67}GaCl_3$ preparations is 0.6 mg/ml and the amount present in 1 µl of new stock $^{67}GaCl_3$ is 5,000 to 12,000 times that of the ^{67}Ga. The results of the ^{111}In experiment indicate that levels of 100 times the amount of Zn to chelate reduce the chelation to 0. It is not surprising then that the ^{67}Ga failed to bind to the B-EDTA-poly-H-IgG when relatively small amounts of chelated protein were used and only 13% of the ^{67}Ga could be chelated by high levels of labeled protein.

IN VITRO STABILITY OF CHELATE LABELED POLY-H-IGG

The stability of the radiometal chelated protein in solution is important when determining the shelf life of the labeled product. Table 16 summarizes the results of an extended stability study using chelate labeled poly-H-IgG. Baseline parameters indicate that virtually 100% of the ^{111}In is chelated to the protein. The low result for the product later incubated at 37°C may be due many things. Incomplete mixing prior to TLC would reduce the amount that appeared to be chelated or contaminants on the TLC plate may have competed with the small amount of protein for the ^{111}In. It should be noted that ^{111}In was virtually 100% chelated when tested at 18 hours for this solution.

The maximum loss of ^{111}In was 12.0 ± 0.9% at 25°C after 3 weeks. The ^{111}In label was very stable at 37°C, losing only 3.4 ± 0.5% of the ^{111}In after 3 weeks. The high stability of the ^{111}In label in solution would allow preparation of the radio-chelated protein several days prior to use or storage of the radio-chelated protein for subsequent use without significant loss of the radiolabel.

TABLE 16. *In Vitro* Stability of ^{111}In-B-EDTA-poly-H-IgG

Time in hours	Percent Bound to B-EDTA-poly-H-IgG (x ± s.d., n=3) 37°C	25°C	4°C
0.33*	79.91 ± 0.64	98.82 ± 0.45	95.63 ± 0.11
18.5	98.66 ± 0.06	98.86 ± 0.04	98.08 ± 0.08
[illegible]	97.49 ± 0.37	97.96 ± 0.01	96.38 ± 0.09
[illegible]	[illegible]	97.86 ± 0.01	96.62 ± 0.13
138.5	97.38 ± [illegible]32	97.56 ± 0.16	96.64 ± 0.30
503	96.55 ± 0.49	88.0 ± 0.92	95.55 ±0.16

* All solutions were incubated at 25°C for 20 minutes to assess baseline parameters prior to temperature study.

3.1.7.2 CHELATE LABELING TECHNIQUES

N;-B-EDTA COUPLING REACTION

This derivative of PAB-EDTA was the first reported by Goodwin *et al.*[107] as a simple, mild chemical modification to introduce the benzyl-EDTA chelate into proteins. Diazotization has been used in the past for affinity labeling of antibodies,[108] for selective modification of enzymes[109,110] and for labeling BSA in immunochemistry.[111] Diazonium salts couple readily with lysyl, tyrosyl and histidyl residues of proteins to yield colored derivatives.[112] With this in mind, this compound was tested to determine the usefulness of diazonium coupling to introduce the chelate label.

Initial labeling studies by Leung[141] with HSA utilized a 10 fold molar excess of N;-B-EDTA to HSA. A variety of conditions were investigated and he concluded that high labeling yields were favoured by low ionic strength and a concentrated HSA solution. Bound chelate to albumin ratios ranged from 1.3 to 5.4:1 and the differences in labeling yield were due mainly to the dependence of the reaction on pH, time, ionic strength and protein concentration. The ideal conditions included an HSA concentration of 50 to 100 mg/ml in 0.15 M NaCl with pH adjusted to 9 after addition of the N;-B-EDTA. In other experiments, Leung *et al.*[34] determined that the labeled residues obtained with a reaction pH of 8.0 were mainly derivatized lysine although the exact site of attachment could not be determined by amino acid analysis.

The protein solutions utilized in these experiments ranged from 1 mg/ml to 20 mg/ml and variable labeling yields were obtained. The reaction conditions used were equivalent to those described above.[141] Table 17 lists the labeling yields as determined by standard $^{111}InCl_3$ assay and therefore only represent the available chelate in contrast to total chelate labeled on the protein.

Unlike Leung's results,[141] there seems to be no trend to correlate the amount of chelate reagent with protein concentration. The difference between the

TABLE 17. Diazotization Reaction Yields.

Chelate to Protein Ratio	Protein Used for Coupling			
	poly-H-IgG 20 mg/ml	BSA 1 mg/ml	MAb-46D 11 mg/ml	MAb-M1A 3 mg/ml
initial	10 : 1	3 : 1	10 : 1	22 : 1
final	0.76 ± 0.33	0.24†	2.7 ± 0.6	6.0 ± 1.2

† Single determination, all others the x ± s.d of 3 to 4 values

poly-H-IgG and MAb-46D at 10:1 chelate : protein is the concentration of protein and conversely to what Leung found, the lower protein concentration produced higher yields (2.7 available chelates per protein molecule as compared with 0.76). Since diazonium compounds couple to free amino groups on lysyl, tyrosyl and histidyl residues an explanation for the difference observed may simply be amino acid composition and conformational structure of the two immunoglobulins. MAb-46D may also have more available residues for labeling. The highest labeling yields were obtained with MAb-M1A at relatively low protein concentration but high chelate : protein ratio. The effect on *in vitro* MAb reactivity was studied using MAb-46D and this is discussed in section 3.1.8. MAb-M1A could not be studied in this manner due to a lack of a suitable antigen preparation for the ELISA.

As well, since these labeling yields represent only the available chelate for ^{111}In binding, the total chelate content could be much higher and there may be 'hidden' chelate molecules on the protein that cannot bind ^{111}In. Leung[34] refers to this when comparing the total chelate present as calculated by ^{14}C labeled N;-B-EDTA to that calculated by the standard ^{111}InCl, assay. Since the available chelate is of greater importance, the labeling yield calculated by the TLC assay is sufficient for the purposes of these studies.

A major disadvantage of using N;-B-EDTA is the significant amount of protein precipitation that occurs upon addition of the chelate reagent. Up to 10% of the protein is precipitated with chelate : protein ratios of 10:1, removing a significant amount of protein from active participation in the reaction. Re-measurement of the protein concentration is also necessary to determine the labeling yield.

The final labeling yield with ^{111}InCl, ranged from less than 3% to 30% for ^{111}InCl, obtained from Medi-Physics and up to 80% using ^{111}InCl, obtained from AECL.

BrAc-B-EDTA COUPLING REACTION

With the problems of precipitation encountered with the N;-B-EDTA reaction, the first consideration was the use of a strong buffer for BrAc-B-EDTA reaction. Meares *et al.*[11] employed a 0.15 M phosphate buffer at pH=8.0 with readjustment to pH=9.0 after addition of the BrAc-B-EDTA. With this in mind, various strengths of phosphate buffer at pH=8.5 were tested to determine which molarity would maintain the protein in solution and not undergo significant pH changes upon addition of the acidic BrAc-B-EDTA reagent (see Table 18). The 0.01 M phosphate buffer easily maintains the protein in solution but is readily subject to pH change upon addition of acid. All other molarities tested maintained pH but were variable in their ability to keep the protein in solution. The buffer of choice is 0.2 M phosphate since it does not cause protein precipitation over a wide concentration range and adequately maintains the pH after addition of BrAc-B-EDTA up to 100:1 chelate : protein for the highest concentration of protein tested.

With increasing molarity and increasing protein concentration, less protein remains in solution. The absolute amount of protein in solution for the lowest concentration tested is much less than that for the highest concentration. This difference is due to the fact that three different stock solutions were used and at each molarity tested the percentage of protein precipitation depended upon the initial protein concentration. With lower protein concentrations, less is precipitated and the results should be analyzed on the percent changes rather than on absolute amounts. Since precipitated protein represents denatured protein and is highly undesirable for these reactions, 0.2 M phosphate buffer at pH=8.5 was chosen for the BrAc-B-EDTA reactions.

The typical yields for the BrAc-B-EDTA reactions are given in Table 19. The major difference to be noted from these results is the minimum percent yield for the 1:1 and 10:1 reactions for MAb-155H.7 compared with these reactions for

TABLE 18. Solubility of Poly-H-IgG in Phosphate Buffer.

Stock Protein*	Strength of Phosphate Buffer, pH = 8.5			
	0.01 M	0.2 M	0.5 M	1.0 M
A) 23	22.6	22.1	10.4	6.2
B) 5	4.9	4.6	4.3	3.8
C) 1.5	1.4	1.3	1.2	0.92

* Concentration in mg/ml

TABLE 19. Reaction Yields of BrAc-B-EDTA Conjugation

	BrAc-B-EDTA : Protein Reaction Ratio						
	poly-H-IgG				MAb-155H.7		
	1:1	10:1	50:1	100:1	1:1	10:1	100:1
Available chelate per protein[1]							
1) by TLC[2]	ND[3]	0.2 ± 0.02	5.5 ± 1.0	12.2 ± 3.1	0.2 ± 0.04	0.6 ± 0.06	7.2 ± 0.6
2) by GF[4]	ND[3]	0.3 ± 0.0	5.3 ± 1.2	10.3†			
Average % yield	ND[3]	1.9 ± 0.1	10.0 ± 2.5	10.9 ± 1.7	21.4 ± 3.2	6.1 ± 0.6	7.2 ± 0.6

1. Available chelate per protein measured on purified protein
2. TLC - thin layer chromatography
3. ND - not detected
4. GF - gel filtration

† Single determination, all others the x ± s.d. of 3 to 8 determinations

poly-H-IgG. In the case of the 1:1 ratio, no detectable yield could be determined for poly-H-IgG whereas the best labeling yield of 21.4% was obtained for MAb-155H.7 for the same ratio. Haloacetamides of the type used here are known to react readily with free sulfhydryl groups on proteins but can also react with free amino goups and other nucleophiles.[45] This can be controlled to some extent with pH and when the chelate : protein ratio is kept low, sulfhydryl goups, are the primary site of attachment.[141] These results suggest that MAb-155H.7 contains a higher free sulfhydryl content than poly-H-IgG. When the chelate ratio is increased, the difference is less notable although at 10:1 the minimum percent yield is still higher for MAb-155H.7 than for the poly-H-IgG preparation.

The minimum percent yield and available chelate ratio refer to the fact that the standard TLC assay used here is only measuring chelates present on the protein that can bind ^{111}In (see section 3.1.7.1). By using a DTNB assay, Yeh[143] was able to determine the number of free sulfhydryl groups present on the HSA preparation he used for labeling studies with BrAc-B-EDTA. The total available chelate : protein ratio was calculated to be in a 3 fold excess to the number of sulfhydryl groups present. As well, at the end of reaction, no free sulfhydryl groups were detectable by the DTNB assay. This evidence suggests that the BrAc-B-EDTA first attacks the available sulfhydryl groups and then reacts with other nucleophiles present.

When the ratio is kept at 10:1 or less, the total available chelate present on the protein is less than 1:1 for both MAb-155H.7 and poly-H-IgG. This is in contrast to the work of Meares *et al.*[58] where an average ratio of 3:1 was obtained with a chelate : protein reaction ratio of 10:1. Only when the chelate : protein ratio is increased to 50:1 or 100:1 is there a significant increase in the total available chelate on the protein.

Labeling yields of no carrier added $^{111}InCl_3$ for the BrAc-B-EDTA labeled proteins ranged from 30% or less for proteins labeled at less than 1:1 available chelate : protein and greater than 95% for proteins containing 1:1 or more available

chelates per protein molecule. Purification of ^{111}In labeled proteins was carried out using the mini-column procedure described in section 3.1.7.1.

IODINATION REACTIONS

Standard iodinating techniques using iodine monochloride (ICl), chloramine-T (Cl-T)[114] and Iodo-gen were compared to determine labeling yield and purity of labeled product.

Both ICl and Cl-T are well known methods for incorporating radioiodine into proteins.[114] In the ICl method, radioactive NaI (Na*I) is equilibrated with ICl to produce *ICl with ICl carrier. Under these conditions, the *I-Cl bond is slightly polarized such that the *I possesses a net positive charge. At neutral pH, this allows incorporation of *I$^+$ into tyrosine residues and some histidine residues in proteins (see Figure 7). The major drawback is the incorporation of a certain amount of non-radioactive I from the ICl carrier reducing the specific activity that can be obtained.

Cl-T is a very popular method of iodination because very high specific activity protein can be produced by this method.[24] Cl-T is the sodium salt of the N-monochloro derivative of p-toluene sulfonamide and in aqueous solution at neutral pH is capable of oxidizing Na*I to form *I$^+$. This incorporates into tyrosine and histidine residues on proteins in the same manner as *I$^+$ from *I-Cl. (Figure 7) Although high specific activity proteins can be produced by this method, proteins are reportedly more easily damaged by Cl-T than ICl.

To overcome the oxidation damage produced by Cl-T and the incorporation of non-radioactive I by ICl, an insoluble oxidizing agent was developed known as Iodo-gen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril).[25] This compound is insoluble in aqueous media and reacts similarly to Cl-T to oxidize Na*I to *I$^+$ (Figure 7). The advantages of this compound are the production of high specific activity proteins with minimal contact with the oxidizing agent and the fact that no reducing agent is required to stop the reaction. The labeling yields obtained by these three methods for

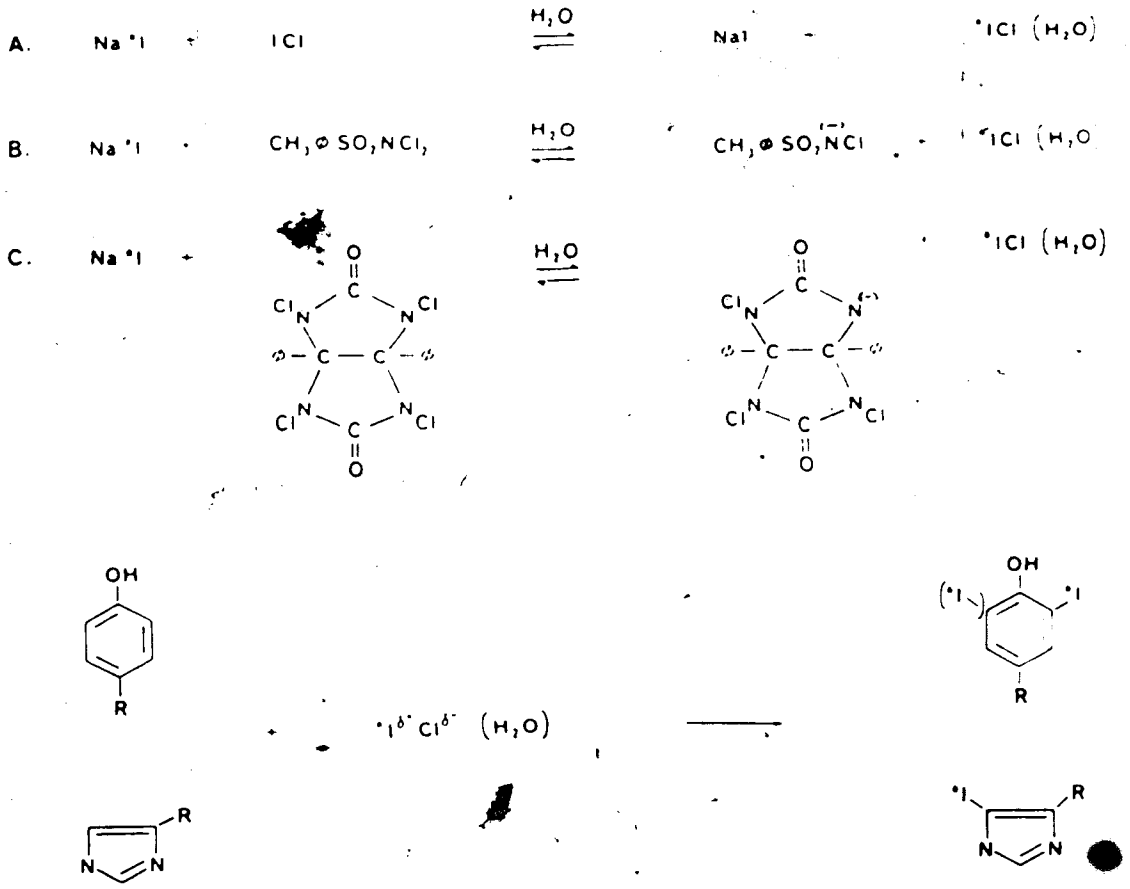


Figure 7. Mechanism of action of A) ICl, B) Chloramine-T and C) Iodo-gen to label tyrosine and histidine with *I. R = $CH_2CH(NH_2)COOH$

MAb-155H.7 are presented in Table 20.

The best labeling yield was produced by Cl-T although this was only 10% higher than either ICl or the best Iodo-gen yield. The time needed to produce this yield for both ICl and Cl-T was 30 seconds and is representative of the rate of radioiodine incorporation for both of these strong oxidizing reagents. Iodo-gen, on the other hand, requires significantly more time to produce comparable yields. This is due to the fact that it is insoluble in the solution being iodinated and is a mild oxidizing agent. The yields were assessed by conventional gel filtration on BioGel P-6DG and although they appear to be less than 30% on the average for all techniques, there were isolated instances of higher yields especially in the Iodo-gen group. The labeling yield is largely dependent upon mixing the solution to maximize contact between the oxidizing agent, the Na*I and the protein. Each reaction tube was hand shaken and the reproducibility from batch to batch for this parameter was hard to maintain. Increasing the yield for ICl or Cl-T could be achieved with longer reaction times but this also increases the risk of protein damage as evidenced by decreased immunoreactivity of the MAb. When Iodo-gen was used with increased amounts of radioiodine, the yield for 1 μg at 5 minutes was on the average 30 to 40%. In the experiments performed here, relatively small tracer amounts of ^{125}I were used and this could also contribute to the low yields obtained.

Since gel filtration was the primary mode of purification, a second method to check the purified proteins for the presence of unbound radioiodine was employed. Trichloroacetic acid (TCA) precipitation is also a well known technique for analysis of iodinated proteins. It is based on the premise that only covalently bound radioiodine will precipitate with the protein. When this technique was applied to the proteins iodinated here, the best results were obtained with the proteins labeled by Iodo-gen. On the average, 98.1 $\pm$ 0.7% of the radioiodine was associated with the precipitated protein. This compares very favourably to the results for ICl and Cl-T, 96 and 93% respectively.

TABLE 20. Radioiodination Labeling Yields for MAb-155H.7 Using Three Different Iodination Methods

Time	Method				
	Iodo-gen			Cl-T	ICl
	1 μg	3 μg	5 μg		
30 sec.	-	-	-	31.7 ± 2.3	20.9 ± 1.5
5 min.	9.7 ± 1.8	39*	20.4 ± 0.2	-	-
10 min.	2.1 ± 0.1	15.3 ± 0.1	21.7 ± 0.9	-	-
30 min.	12.8 ± 0.1	23.1 ± 1.1	21.9*	-	-

* Single determination, all others are x ± s.d., n = 3.

Although Cl-T produced the best labeling yields in this set of experiments, proteins iodinated with Iodo-gen produced better preparations with regard to quality control.

For routine iodination, Iodo-gen was used in a ratio of 1 μg of Iodo-gen per 100 μg of MAb-155H.7 and 5 μg per 100 μg of poly-H-IgG. The centrifuged column procedure was successfully adapted and used routinely to obtain iodinated proteins in concentrated form (100 to 200 μl) with good purity and protein recovery.

3.1.8 *IN VITRO* TESTING OF LABELED PROTEINS

3.1.8.1 ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

The effects of iodination and the chelate reactions were tested in ELISA against the appropriate antigens to determine how these procedures altered the binding of the labeled MAb and how chelation or iodination compared in their effects on the MAbs. The effect of the N;-B-EDTA chelate was compared to iodination on MAb-46D only and the ELISA was carried out at the end of reaction time. Reaction time versus MAb *in vitro* reactivity was more extensively studied for MAb-155H.7 with the BrAc-B-EDTA chelate compared to the iodination techniques examined here. The results for MAb-46D are presented in Table 21.

In this case, both the chelation and iodination reactions had significant effects on the *in vitro* binding reactivity of MAb-46D. Reduction of reactivity was about 50% for both reactions and although the chelate reaction appears to have less damaging effect the difference between these numbers is not significantly different to support one method over the other. In Table 22, the effects of the BrAc-B-EDTA chelate reaction are summarized for MAb-155H.7 with respect to time of reaction.

The control data for this experiment was MAb-155H.7, at the same concentration as for the 10:1 and 100:1 chelate reactions, but without addition of the chelate reagent. This was to test the reaction conditions for effect on the MAb in the absence of the chelating agent. These results do indicate that the MAb is sensitive to the reaction

TABLE 21. ELISA Activity of Radioiodinated and Radiochelated MAb-46D

	Unlabeled MAB-46D	^{111}In-B-EDTA-MAb-46D	B-EDTA-MAb-46D	^{125}I MAB-46D
% Activity Retained ($\bar{x} \pm$ s.d., n = 3)	100	57.5 ± 29.3	53.5 ± 17.5	46.3 ± 13.6

TABLE 22. Effect of BrAc-B-EDTA Reaction Conditions on *In Vitro* Binding Activity of MAb-155H.7

Time (minutes)	Stock* MAb-155H.7 pg / A_{405}	Stock* MAb-155H.7 % of stock	Control* † MAb-155H.7 pg / A_{405}	Control* † MAb-155H.7 % of stock	10 : 1* Chel.:MAb pg / A_{405}	10 : 1* Chel.:MAb % of stock	100 : 1* Chel.:MAb pg / A_{405}	100 : 1* Chel.:MAb % of stock
0	593	100	-	-	-	-	-	-
15	-	-	690	85.9	505	117	772	76.8
30	-	-	712	83.3	610	85.9	926	64.0
60	-	-	716	82.8	1009	58.7	1133	52.3
120	-	-	757	78.3	1340	44.2	1240	47.8

* mean of 4 to 9 replicates

† control consists of MAb-155H.7 in 0.2 M phophate buffer, pH = 8.5 without chelating agent

conditions employed and loses approximately 20% of its *in vitro* binding reactivity. Other reaction conditions explored by Leung[44] showed that no reaction occurred at 4°C and both Leung[44] and Yeh[93] favoured elevated temperatures for the BrAc-B-EDTA reaction. When the chelating agent is present a further 20 to 25% reduction of *in vitro* binding reactivity is observed after a 60 minute reaction time with the maximum reduction being 30 to 35% after 2 hours. The net reduction of binding reactivity is 44% for the 10:1 reaction and 48% for the 100:1 reaction. This is comparable to the 53% retained for the 10:1 N;-B-EDTA reaction with MAb-46D. Both chelate reactions reduced considerably the *in vitro* binding reactivity of the MAbs tested but the BrAc-B-EDTA reaction was preferred due to its ease of execution. The effect of the various iodination reactions on MAb-155H.7 binding reactivity is shown in Table 23.

MAb-155H.7 has proven to be very sensitive to all three iodination techniques but especially to ICl and Cl-T. Thirty second exposure to either one of these reagents reduces *in vitro* binding reactivity by 80%. Iodo-gen was more easily controlled by manipulating both exposure time and the concentration of Iodo-gen. The best amount and time was 1 μg of Iodo-gen and 5 minutes of exposure. From analysis of the yield, however, the percent incorporation of radioiodine varied from 10% for tracer quantities of ^{123}I (2 MBq) to only 30 to 40% for higher quantities of ^{123}I (20 to 40 MBq). When ^{131}I (20 to 40 MBq) was used for iodinating MAb-155H.7 for the scintigraphic studies, it was found that the *in vitro* binding reactivity was completely lost after 3 days. This is most probably related to radiation induced damage generated by the β component of ^{131}I. For ^{111}In, the retained *in vitro* binding reactivity remained stable for several days both at tracer (2 MBq) quantities and at higher levels (37 to 74 MBq). MAb-46D was more resistant to both iodination reaction conditions and radiation induced damage. The percent retained binding activity for this MAb was 46% after a 30 minute exposure to 8 μg of Iodo-gen. These two MAbs demonstrate the need to individualize reaction conditions for both iodination and chelation reactions.

TABLE 23. Percent *In Vitro* Binding Activity Retained After Radioiodination for MAb-155H.7

Amount of Iodo-gen	Iodo-gen Reaction Time 5 min.	Iodo-gen Reaction Time 10 min.	Iodo-gen Reaction Time 30 min.	ICl Reaction Time 30 sec.	Cl-T Reaction Time 30 sec.
1 μg	61.3	36.4	40.4	20.0	18.4
3 μg	41.6	22.7	28.3	-	-
5 μg.	24.2	24.5	4.8	-	-

All values the mean of 3 determinations.

The major advantage of the chelation reaction over the iodination reaction is the preparation of the chelate-protein complex in advance of the actual radiometal labeling. In this way, a stock solution of the protein can be labeled with the chelate and stored at -80°C until needed. Compared to iodination, the technique is more convenient and reduces the radiation exposure of the operator. A second distinct advantage is the availability of several radioisotopes for radiolabeling which possess more suitable physical decay properties, viz. ^{67}Ga and ^{111}In. Therefore, although iodination and chelation reactions have similar effects on the proteins tested here, the chelation reaction offers many advantages over iodination and would be the reaction of choice.

3.1.8.2 CELLULAR ASSAYS

Two types of cellular assays, the whole cell ELISA and live cell uptake, were used for further *in vitro* characterization of the labeled proteins.

In the whole cell ELISA, used only for MAb-155H.7, murine and human tumour cell lines were grown in 96 well micro-titre plates and fixed using gluataraldehyde. The rest of the ELISA procedure was carried out comparing unlabeled MAb-155H.7 to the various labeled forms of MAb-155H.7. Figure 8 graphically demonstrates the results obtained expressed in terms of unlabeled MAb binding.

All cell lines tested showed some association of MAb-155H.7 in the unlabeled and chelate labeled forms. Unfortunately, for this particular assay, the iodinated MAb-155H.7 had retained only 5% of its *in vitro* binding activity when tested in the standard ELISA using Tβ-HSA. This could account for the low binding shown in the cellular ELISA. The effect of increasing amounts of chelate on the MAb is clearly seen by a decrease in association of the MAb with the cells. This is particularly true for the human cell lines, LoVo and SW1116. The other human cell line, MIA-PACA, as well as the murine cell lines, EMT-6 and RI, show moderate decreases with increasing chelate.

For LoVo and SW1116, the 'binding site' on MAb-155H.7 for the cell may be very close to or in the same site as the chelate label thus explaining the decrease in binding as the increasing chelate bound alters this binding site. The murine cell lines and

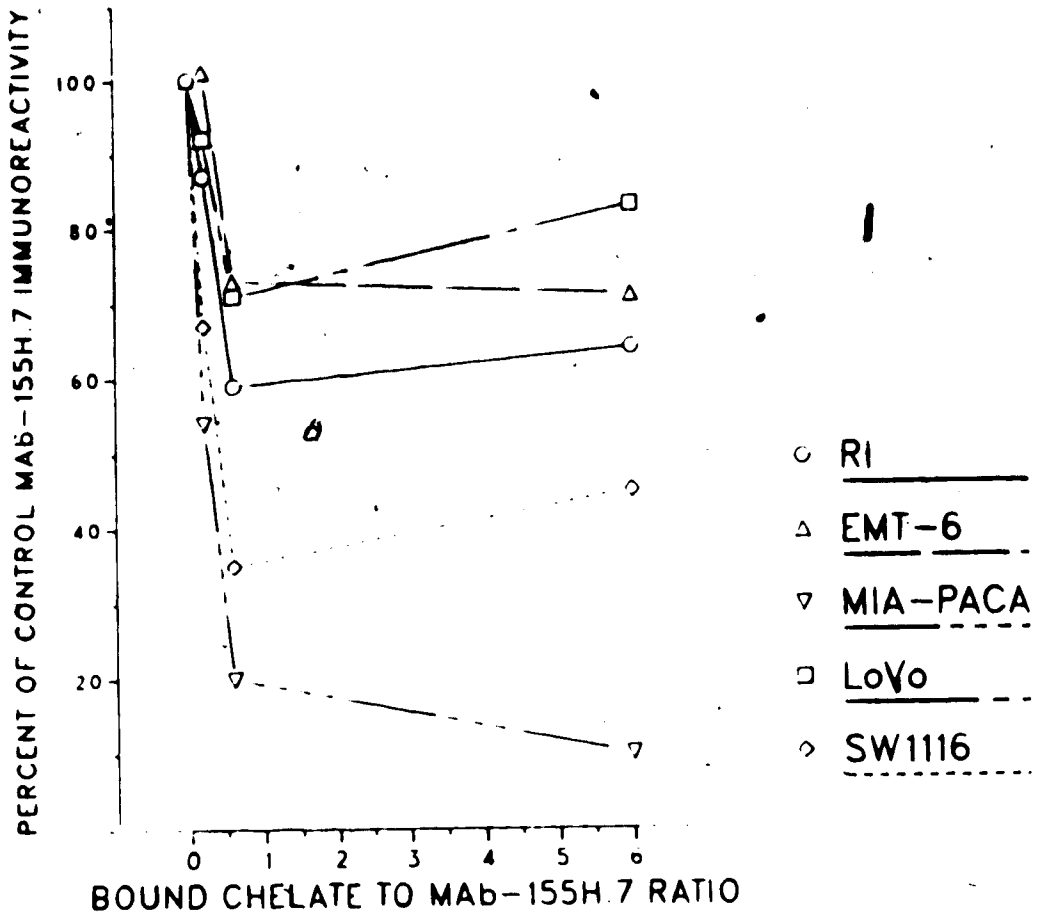


Figure 8. Whole cell ELISA of ^{111}In-B-EDTA-MAb-155H.7 on murine tumour cells, RI and EMT-6, and human tumour cells, MIA-PACA, LoVo and SW1116.

the third human cell line, MIA-PACA, may have a 'receptor' for MAb-155H.7 that requires a different part of the MAb which is not affected as severely by increasing the chelate label. Since MAb-155H.7 was generated against the T-antigen it might be tempting to say that these cell lines possess this antigen and this is what the MAb is binding to. However, EMT-6 cells do not posess this structure and the status of MIA-PACA with regard to the T-antigen is unknown. RI cells have demonstrated the T-antigen on their surface and as both LoVo and SW1116 are derived from colorectal carcinomas it is possible for these cell lines to have the T-antigen. The evidence presented by this data however is not conclusive for the presence or absence of the T-antigen on these cells. As well, glutaraldehyde fixation is know to affect the expression of cell membrane antigens. It can unmask previously hidden antigens or cleave exposed antigens from the cell surface thereby altering the interaction of the MAb with the cell.

The live cell uptake studies were performed on murine tumour cell lines only, due to their ease of handling and reduced biological hazard as compared to the human cell lines. Figure 9 represents the percent uptake of each labeled protein per 10^6 cells with ^{111}In-citrate and Na^{125}I as radiolabel controls. The cells were suspended in serum-free media (RPMI 1640) after being washed 3 times in RPMI-1640. The labeled proteins were diluted in PBS without the addition of 1% BSA. From these results, a general trend of decreasing uptake with increasing amount of chelate on MAb-155H.7 is evident for EMT-6 and EL4 cells. For RI and TA3/Ha cells, the uptake remains the same or increases slightly for increasing chelate labeled to MAb-155H.7. In all cases, the uptake of ^{111}In-B-EDTA-MAb-155H.7 is greater than ^{111}In-citrate. As well, iodinated MAb-155H.7 shows similar or slightly better uptake when compared to ^{111}In-B-EDTA-MAb-155H.7 with uptake of Na^{125}I by all cell lines negligible. The uptake of poly-H-IgG is somewhat surprising since this is a non-specific immunoglobulin preparation and in some cell lines, such as TA3/Ha, is almost identical to that of the labeled MAb. The whole cell ELISA and standard ELISA with Tβ-HSA showed no binding of poly-H-IgG relative to MAb-155H.7. This would suggest that the binding observed on these cell lines is

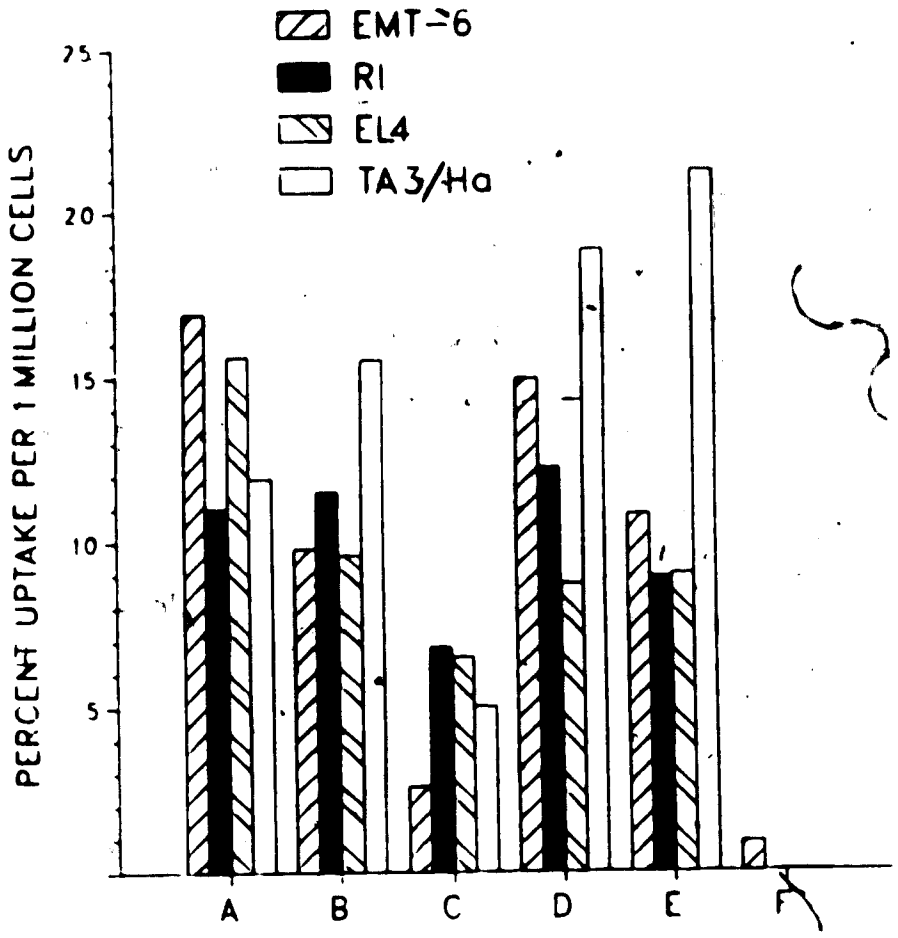


Figure 9. Murine tumour cell uptake of radiolabeled MAb-155H.7 expressed as percent uptake per 10^4 cells.
A = ^{111}In-B-EDTA-MAb-155H.7 (0.5:1 chelate:MAb), B = ^{111}In-B-EDTA-MAb-155H.7 (6:1 chelate:MAb), C = ^{111}In-citrate, D = ^{125}I-MAb-155H.7, E = ^{125}I-poly-H-IgG, F = Na^{125}I.

nonspecific since the uptake was performed in the absence of carrier proteins (FBS or BSA) or that the poly-H-IgG contained significant amounts of anti-T antibodies. The presence of anti-T in a polyclonal preparation of human IgG is virtually insignificant as Springer[1] indicates that most of the human anti-T present in serum is IgM and not IgG. The second point against this uptake being due to anti-T in the polyclonal preparation is that if the amount of anti-T IgG is calculated and then the amount of iodinated anti-T is calculated, the resulting concentration is insignificant relative to the other non-anti-T IgG in the preparation. Another possibility is the presence of immunoglobulins of undefined origin in the poly-H-IgG preparation that are binding to other receptors on the cell surface and are therefore taken up to the same extent as the labeled MAbs. The most likely explanation is the non-specific association of the labeled proteins to the cells due to the absence of carrier proteins and the binding of the IgG's to Fc receptors on the cells.

The effect of the presence or absence of BSA and FBS in the preparations used for the cell uptake was studied using the TA3/Ha tumour cells. These results are presented in Table 24 for the iodinated MAb-155H.7 and chelate labeled MAb-155H.7 with 6 chelates per protein molecule. The presence of both BSA and FBS severely inhibits the binding of MAb-155H.7 to TA3/Ha cells. When one or the other is present the inhibition is less than when both are present but still represents a significant reduction in the binding of the labeled MAbs. BSA and FBS do not cross-react with MAb-155H.7 so the inbition of binding must be through another mechanism or could be just due to the fact that these labeled proteins are associating nonspecifically to the cells in the absence of BSA or FBS. This nonspecific association could be blocked by the presence of BSA or FBS.

Overall, the results do not support the specific association of MAb-155H.7 to any of the cells tested in either the whole cell ELISA or the live cell uptake. Tumour cells grown *in vitro* are in artificially controlled media and as such cannot be expected to mimic the three dimensional response that would be observed *in vivo*.

TABLE 24. Percent Uptake of 3 ng of ^{125}I-MAb-155H.7 and ^{111}In-B-EDTA-MAb-155H.7 (6:1) per 10^4 TA3/Ha Cells in the Presence and Absence of FBS and BSA

Cells Incubated in:	MAb-155H.7 Diluted in: PBS		1% BSA/PBS	
	^{111}In-MAb-155H.7	^{125}I-MAb-155H.7	^{111}In-MAb-155H.7	^{125}I-MAb-155H.7
PBS	9.1 ± 1.1	12.6 ± 1.4	2.5 ± 0.2	0.8 ± 0.06
PBS + 10% FBS	1.6 ± 0.1	2.9 ± 0.8	0.4 ± 0.1	0.5 ± 0.06
RPMI	7.4 ± 1.0	12.6 ± 0.4	0.9 ± 0.06	1.0 ± 0.2
RPMI + 10% FBS	1.3 ± 0.08	2.0 ± 0.4	0.3 ± 0.06	0.3 ± 0.06

All values the $\bar{x}$ ± s.d. of 3 determinations.

3.1.8.3 RBA AND HISTOLOGICAL SCREENING

Along with the testing in *in vitro* cell systems, a radioacitive binding assay (RBA) using Tβ-HSA and T-synsorb was investigated. Immulon Remova-well strips (Dynatech) were coated with 1 μg per well with Tβ-HSA. Approximately 0.5 to 1.0 mg of Tβ- and Tα-synsorb (S) (ChemBioMed) were weighed into micro centrifuge tubes. The amount of Tβ and Tα was calculated from the stated concentration on the label. To each of these was added the labeled proteins used in the live cell uptake with a standard ELISA being conducted in parallel for comparison purposes. All incubations were for one hour at room temperature Results for all assays are presented in Table 25.

data indicates no difference in the amount bound with different labeling techniqu this is contradictory to what was obtained with the corresponding ELISA. Two possibilities present themselves to explain this data. On the one hand, the labeling technique may not be interfering with the binding of the MAb to its antigen, but may alter some part of the rest of the MAb and reduce the binding of the enzyme conjugated antibody used in the standard ELISA. On the other hand, the specific activity of the MAbs tested was relatively low to accomodate the cell uptake study. This results in a low activity bound to the antigen on the solid phase for the RBA. With low activity, the confidence level in determining the amount bound to each well or synsorb is reduced and significant errors can be introduced in this calculation.

In this case, although the chelate labeled MAb-155H.7 retained practically 100% of its binding to Tβ by ELISA as compared to only 24% for the radioiodinated MAb-155H.7, the percent bound by RBA was equivalent for all three proteins. The percent bound reported here has been corrected for ^{111}In-citrate and Na^{125}I binding to the wells in the absence of protein. As well, the poly-H-IgG shows no binding to Tβ-HSA in either the RBA or the ELISA, another indication that there are no anti-T antibodies in this preparation. The results obtained with Tβ-S and Tα-S represent binding of MAb-155H.7 to a commercial source of the synthetic T-antigen. Again, no correlation to the type of label can be seen in the binding to Tβ-S. The radioiodinated preparation of

TABLE 25. RBA and ELISA Data of Radioiodinated and Radiochelated MAb-155H.7 and Radioiodinated Poly-H-IgG on Various Forms of the T-antigen

Protein	ELISA	RBA on Tβ-HSA		RBA on Tβ-S*		RBA on Tα-S**	
	% Bound of MAb-155H.7†	% Bound	ng bound	ng MAb / nmole Tβ	ng MAb / mg Tβ-S	ng MAb / nmole Tα	ng MAb / mg Tα
MAb-155H.7†	100	-	-	-	-	-	-
^{111}In-MAb-155H.7 (0.6:1)	97.5	3.1	0.5	0.15	0.09	0.58	0.16
^{111}In-MAb-155H.7 (6:1)	81.0	2.2	0.4	0.15	0.09	0.14	0.04
^{125}I-MAb-155H.7	23.9	3.8	0.6	0.47	0.27	3.28	0.89
^{125}I-Poly-H-IgG	1.3	0	0.0	0.0	0.0	0.0	0.0

* Tβ-Synsorb - 0.58 μmoles of Tβ per g of Synsorb, 1 mg = 0.58 nmoles

** Tα-Synsorb - 0.27 μmoles of Tα per g of Synsorb, 1 mg = 0.27 nmoles

† MAb-155H.7 = Unlabeled MAb-155H.7
All values the mean of 2 determinations

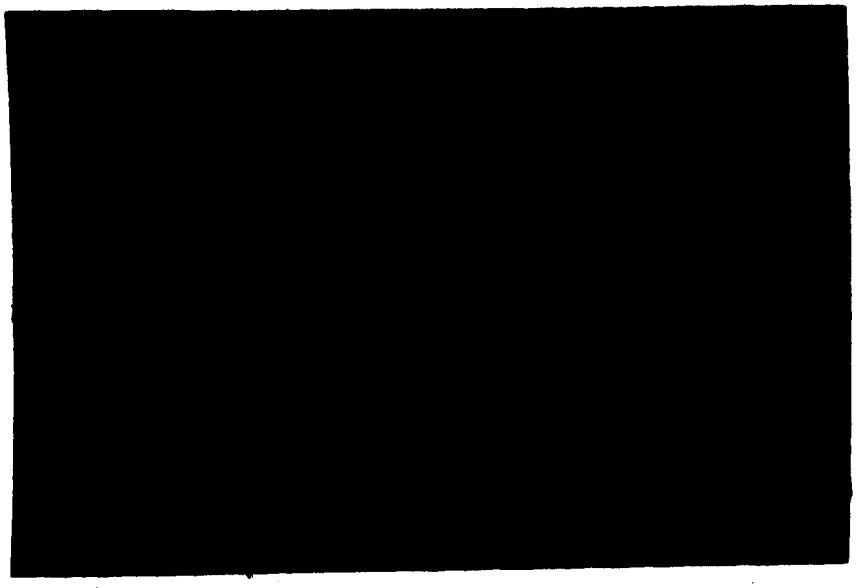
MAb-155H.7 shows higher binding to Tβ-S than to the Tβ-HSA used in the standard ELISA. The chelated preparation of MAb-155H.7 shows significantly reduced binding to Tβ-S when compared to the radioiodinated preparation which is exactly opposite to that obtained with the standard ELISA. Some correlation for the chelate labeled MAb-155H.7 can be seen in the binding to Tβ-S where the higher chelate substituted MAb-155H.7 shows reduced binding. However, the radioiodinated preparation shows a 7 fold increase in binding to Tα-S than to Tβ-S and is significantly higher in binding to Tα-S than the chelated MAb-155H.7. The difference observed in the binding indicates that the Tβ-HSA antigen is different from the commercially available T-synsorbs and in this case the MAb-155H.7 binds better to the Tα-S than to the Tβ-S. It should be noted here that MAb-155H.7 does cross react with Tα-HSA as observed in ELISA data not included here.

For this antibody, then, the standard ELISA utilizing Tβ-HSA gives the most useful information for determining the percent *in vitro* binding retained by the labeled antibodies. RBA using Tβ-HSA or the commercial T-synsorbs do not give a true indication of the quality of labeled MAb obtained with these labeling techniques.

Histological studies of the various labeled MAb-155H.7 preparations compared to unlabeled MAb were conducted using human adenocarcinoma tissue sections. MAb-155H.7 shows strong binding to human adenocarcinoma tissue in this assay. Figures 10 to 12 illustrate the binding of the various labeled MAb-155H.7 preparations to these sections. There was no difference seen in the binding of the various preparations compared to unlabeled MAb-155H.7, as judged by an independent pathologist, Dr. D. Willans (Department of Pathology, General Hospital). These preparations were identical to those used in the whole cell ELISA, where the radioiodinated MAb-155H.7 had very low reactivity by the standard ELISA.

The various results presented here for the *in vitro* assays of MAb-155H.7 suggest that there is very little correlation between all the techniques used for quality control of the antigen binding activity of the MAb. The RBA using various forms of the T-antigen showed no reproducible pattern of MAb binding. RBA on Tβ-HSA did not differentiate

A.



B.

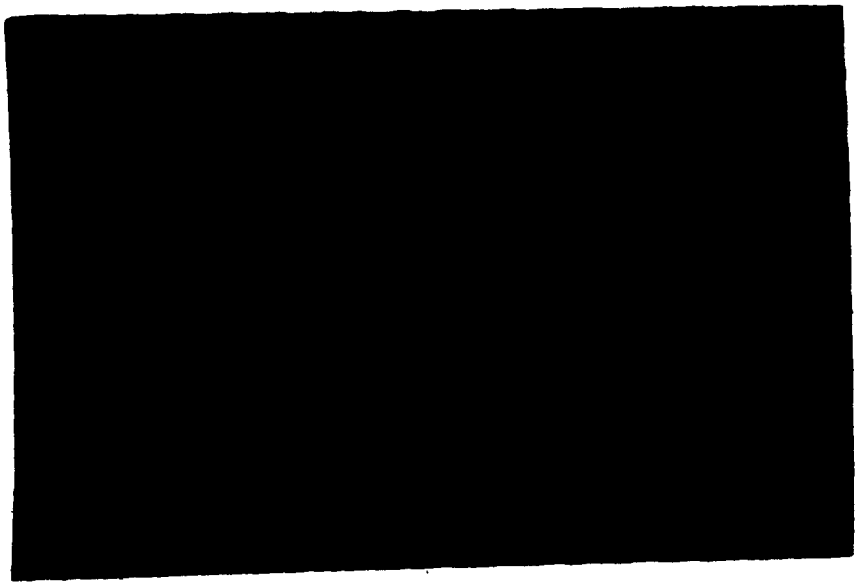
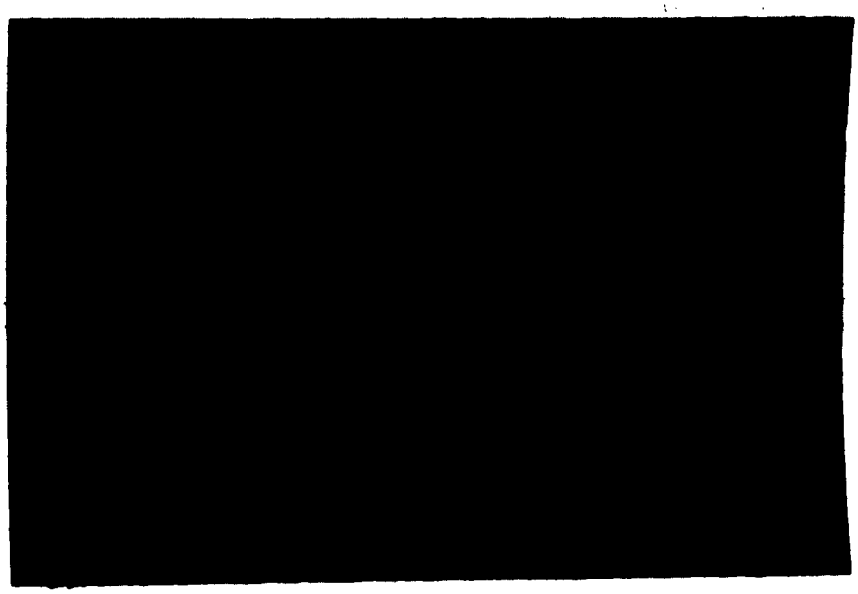


Figure 10. Staining of adenocarcinoma sections with mock radiolabeled MAb-155H.7. A = unlabeled-MAb-155H.7 control, B = unlabeled poly-H-IgG control; DAB (MAb associated stain) is brown; hematoxylin counterstain for nuclei is purple.

C.



D.

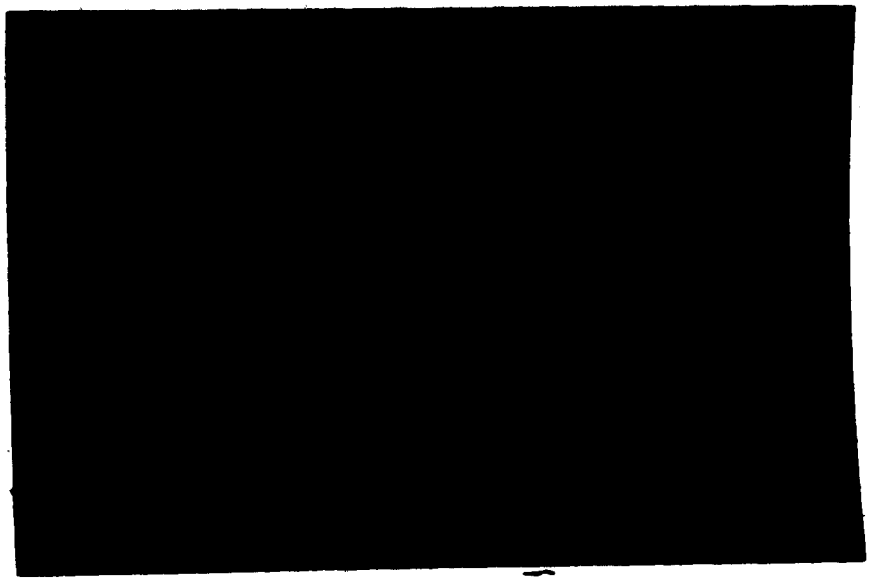


Figure 11. Staining of adenocarcinoma sections with mock radiolabeled MAb-155H.7. C = B-EDTA-MAb-155H.7 (0.1:1 chelate:MAb). D = B-EDTA-MAb-155H.7 (0.6:1 chelate:MAb); DAB (MAb associated stain) is brown; hematoxylin counterstain for nuclei is purple.

E.

F.

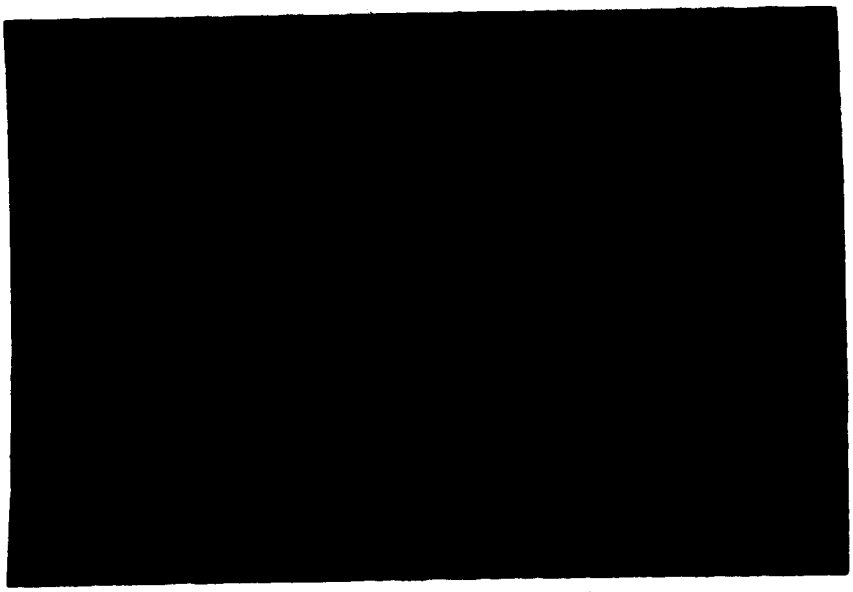


Figure 12. Staining of adenocarcinoma sections with mock radiolabeled MAb-155H.7. E = B-EDTA-MAb-155H.7 (6:1 chelate:MAb). F = mock radioiodinated MAb-155H.7; DAB (MAb associated stain) is brown; hematoxylin counterstain for nuclei is purple.

between radioiodine and radiochelate labeling. The RBA on the synthetic T-synsorbs was confusing, as the MAb bound more strongly to Tα-S and the radioiodinated MAb bound better than the radiochelated MAb. The histology results also showed no difference between radioiodine and radiochelate labeling on the binding of the MAb to the adenocarcinoma sections. The cell uptake studies were inconclusive as well, due to the nonspecific nature of the association of the radiolabeled MAbs and the poly-H-IgG to the cells. The whole cell ELISA did show some decreasing trend of binding for the radiochelated MAb preparations but conclusive results of specific binding under the conditions used were not produced. For this particular MAb, the standard ELISA on the antigen used for immunization appears to give the best indication of the effects of the labeling techniques on the ability of the MAb to bind to its antigen.

3.1.9 *IN VIVO* TESTING OF LABELED PROTEINS

3.1.9.1 BIODISTRIBUTION STUDIES IN NORMAL MICE

DISTRIBUTION OF $Na^{125}I$ AND ^{111}In-CITRATE

The distribution of both $Na^{125}I$ and ^{111}In-citrate was studied in normal Balb/c mice in order to illustrate the distribution of these tracers in unbound form to compare to the labeled protein distribution. Metabolism of the labeled proteins may release the radioactive label and return it to the circulation. The released radiotracer is then important in determining the distribution observed. Ideally, no metabolic release of the radiotracer is desired as this then leaves the injected protein with no tracer to determine its fate.

The results of the distribution of $Na^{125}I$ and ^{111}In-citrate are summarized graphically in [illegible] tissue to blood ratios. Percent of injected dose per gram of tissue and percent of injected dose per organ data are summarized in Appendix 1. The blood clearance of both tracers is very rapid but $Na^{125}I$ is removed more rapidly than ^{111}In-citrate. This is probably due to specific uptake of the ^{125}I in

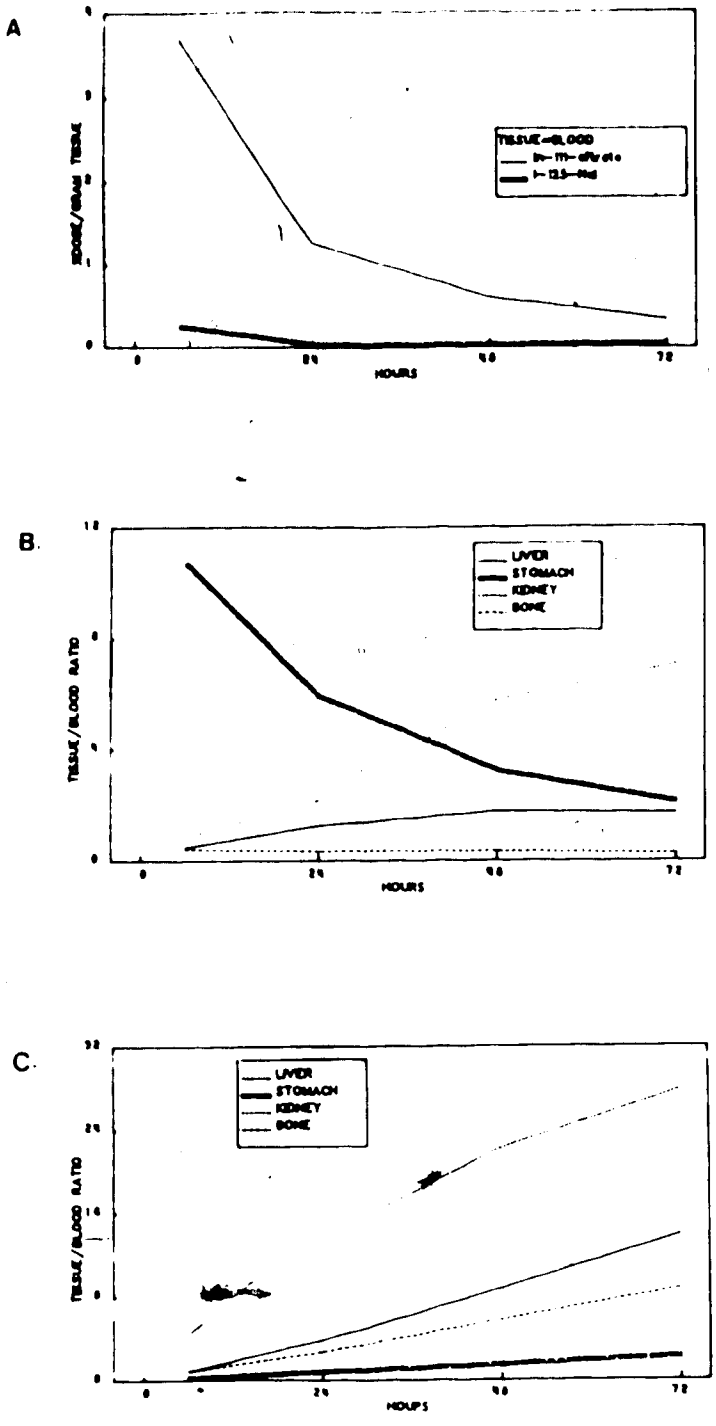


Figure 13. A) Percent of injected dose per gram of blood for $Na^{123}I$ and ^{111}In-citrate and selected tissue to blood ratios for B) $Na^{123}I$ and C) ^{111}In-citrate in normal Balb/c mice. All points the mean of 5 animals with s.d. less than 15%.

the thyroid, salivary glands, stomach and liver all of which are known to concentrate iodine by specific methods. The tissue to blood ratio for the thyroid clearly demonstrates the avidity of this organ for iodine. The level of iodine in the thyroid increases until 48 hours and then declines indicating release of the radioiodine back to the circulation either as free iodine or bound to thyroid hormones. ^{111}In-citrate remains in the blood longer than Na^{125}I and is most likely bound to transferrin.

The kidney, liver and bone are major sites of ^{111}In accumulation with the liver and bone contributing to the transferrin mediated sequestration of ^{111}In and the kidney the site of excretion of the citrate chelate of ^{111}In.

Analysis of the percent of the injected dose remaining (see Appendix 1) at the various time intervals shows a marked difference in the amount of ^{125}I and ^{111}In in these animals. At 6 hours, 87% of the ^{125}I has been excreted and remains relatively constant thereafter to 72 hours while only 50% of the ^{111}In has been excreted at 6 hours and increases to 65% at 72 hours.

DISTRIBUTION OF ^{125}I- AND ^{111}In-PAB-EDTA

Distribution of the dual labeled PAB-EDTA was performed in normal Balb/c mice to establish a means of comparison to the distribution of labeled proteins similar to that for Na^{125}I and ^{111}In-citrate. It was hoped that the iodine label on the phenyl ring of the derivatized EDTA would give a second tracer for the chelating agent in addition to the ^{111}In. The results are presented graphically in Figures 14a and 14b for selected organs over the time periods studied. The percent of injected dose per gram of tissue and percent of injected dose per organ data are summarized in Appendix 2.

Both tracers are cleared very rapidly from the blood and by 24 hours are less than 0.05% of the injected dose per gram of blood. High levels of both isotopes are present in the kidney and liver, the major organs of accumulation. DTPA chelates of ^{111}In and ^{99}Tc^{m} are known to clear rapidly from the circulation by renal excretion. The increased levels of ^{125}I as compared to ^{111}In in the liver, stomach and thyroid are

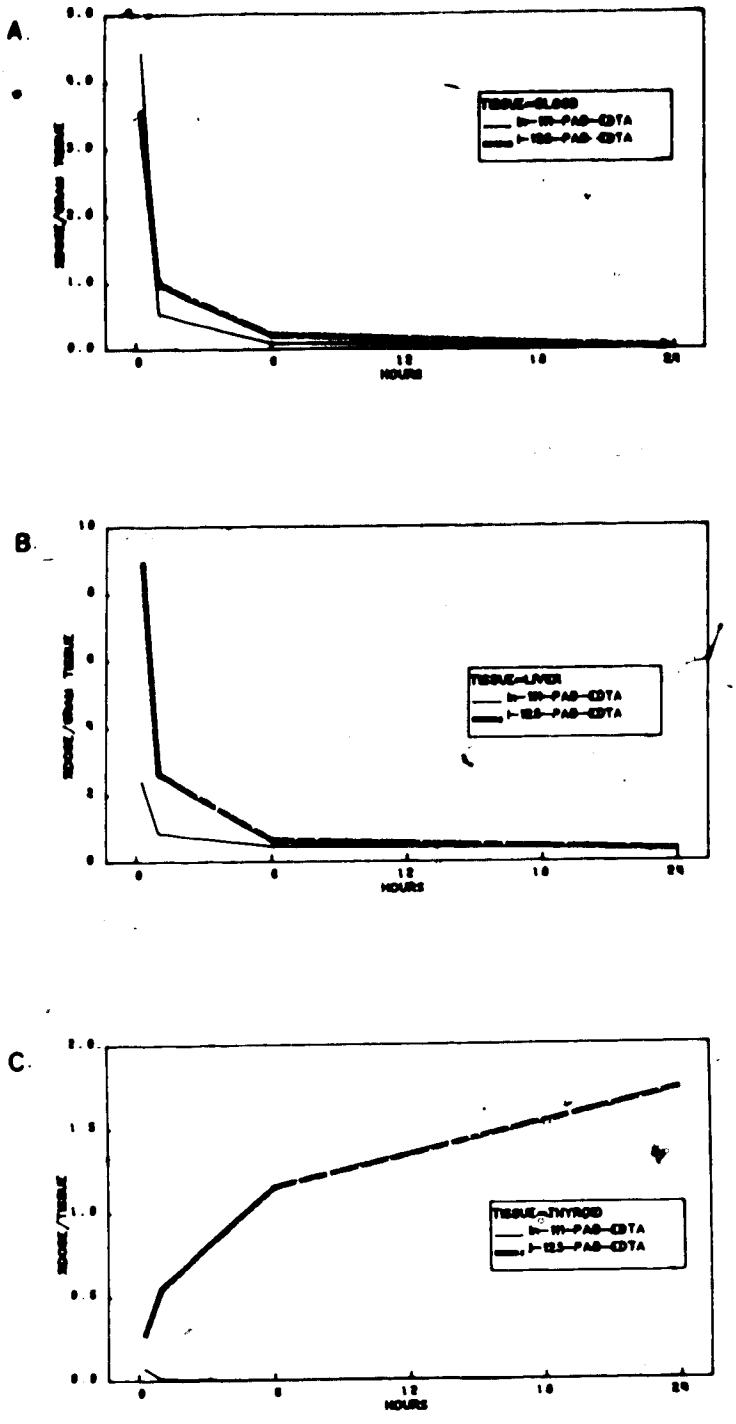


Figure 14a. Percent of injected dose per gram of A) blood and B) liver and C) percent of injected dose per total thyroid for ^{111}In and ^{125}I from dual-labeled PAB-EDTA. All points the mean of 4 animals with s.d. less than 15%.

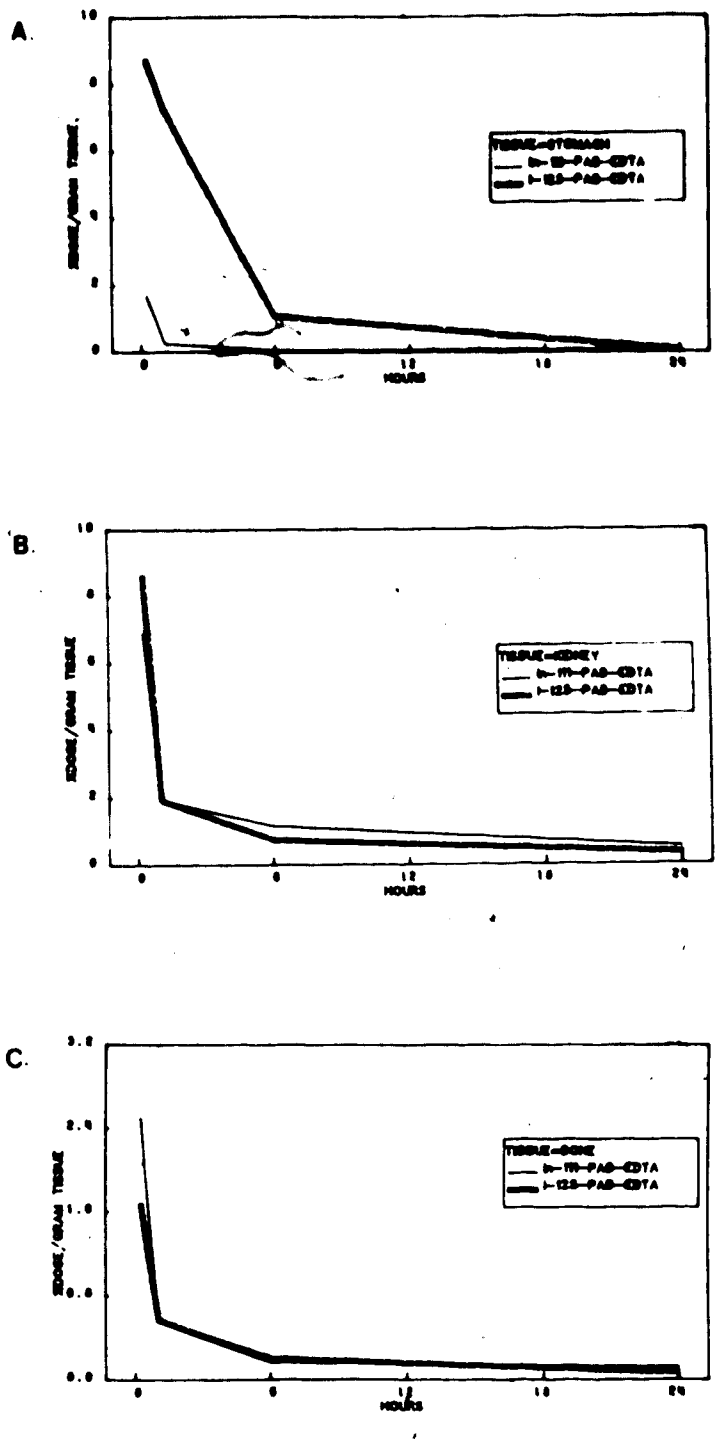


Figure 14b. Percent of injected dose per gram of A) stomach, B) kidney and C) bone for ^{111}In and ^{125}I from dual-labeled PAB-EDTA. All points the mean of 4 animals with s.d. less than 15%.

indicative of the rapid dehalogenation of the compound. The amount of metabolism shows that ^{123}I labeled to the phenyl ring of derivatized benzyl-EDTA is not a good marker to trace the fate of the chelate itself. The high levels in the liver at 15 minutes probably represents a combined pool of unmetabolized chelate and free radioiodine not yet released to the blood. The uptake in the thyroid and stomach would suggest that the metabolized ^{123}I is in the form of free radioiodide. By 6 hours, the dehalogenation process has reached equilibrium and the pattern of distribution of the two radioisotopes is virtually identical except for the radioiodide sequestered in the thyroid. The amount of ^{111}In present in the liver is much lower at 24 hours compared to ^{111}In-citrate indicating the higher stability of the derivatized benzyl-EDTA chelate and its resistance to transchelation to transferrin *in vivo*. This stability of the PAB-EDTA-^{111}In complex is also supported by the *in vitro* studies with transferrin (see section 3.1.3.2).

Bone uptake of ^{111}In is low and both radioisotopes are cleared in parallel from this organ providing more evidence for the *in vivo* stability of the PAB-EDTA-^{111}In complex. The percent dose remaining (see Appendix 1) calculations show a rapid decrease over 24 hours with ^{111}In being excreted at a slightly faster rate than ^{125}I. The retention of ^{125}I is probably due to its metabolism from the chelate and uptake in organs such as the thyroid and stomach. This compound has a unique distribution and can easily be distinguished from the distribution of ^{111}In-citrate. The parallel excretion of both ^{111}In and ^{125}I indicate the stability of the chelate in the presence of transferrin and although there is some release of ^{125}I, the dual label technique used here shows that ^{111}In remains with the chelate when injected *in vivo*.

DISTRIBUTION OF ^{111}In- AND ^{125}I-B-EDTA-POLY-H-IGG

A preliminary distribution of the chelate labeled poly-H-IgG was compared to iodinated poly-H-IgG in Balb/c mice. Figures 15a and 15b illustrate results for selected tissues over the time period studied with percent of injected dose per gram of tissue and per organ data summarized in Appendix 1.

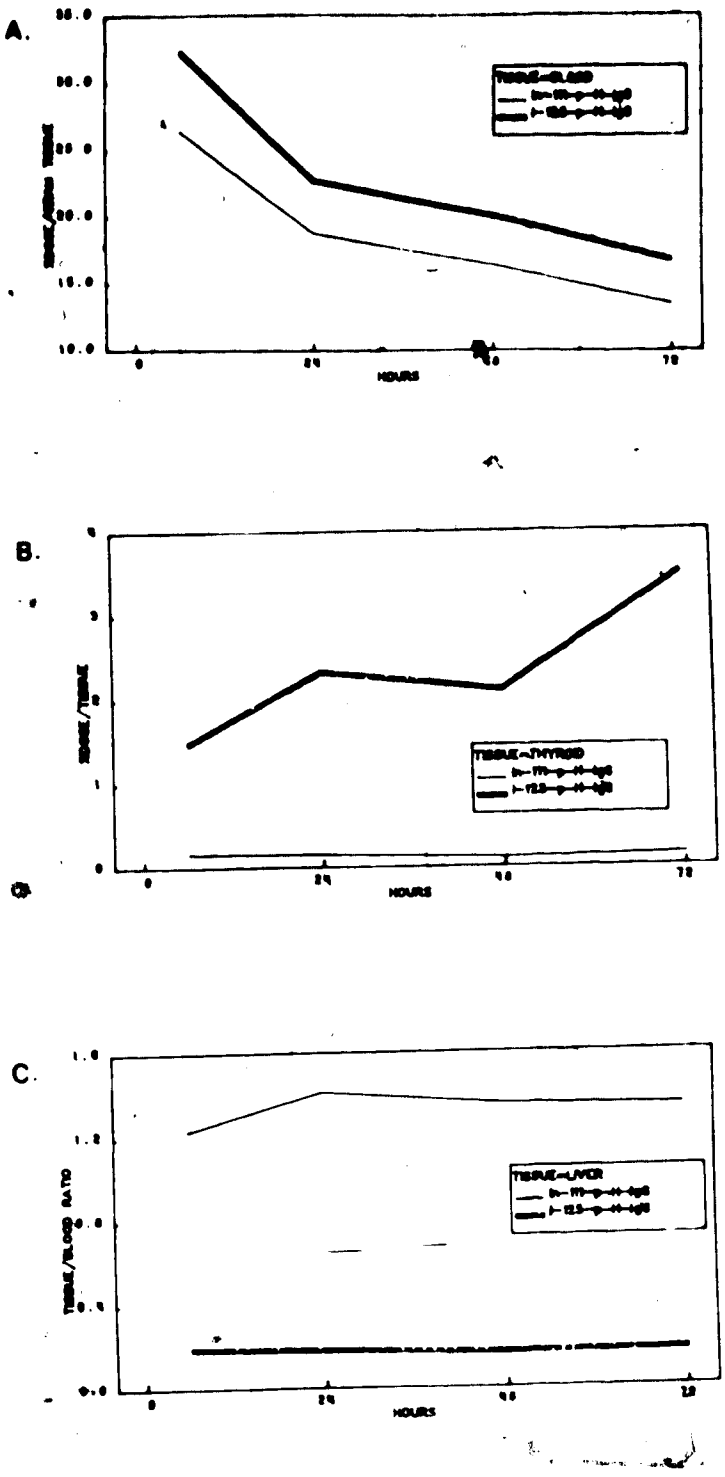


Figure 15a. A) Percent of injected dose per gram of blood, B) percent of injected dose per total thyroid and C) liver to blood ratio for ^{111}In-B-EDTA-poly-H-IgG and ^{125}I-poly-H-IgG. All points the mean of 5 animals with s.d. less than 20%.

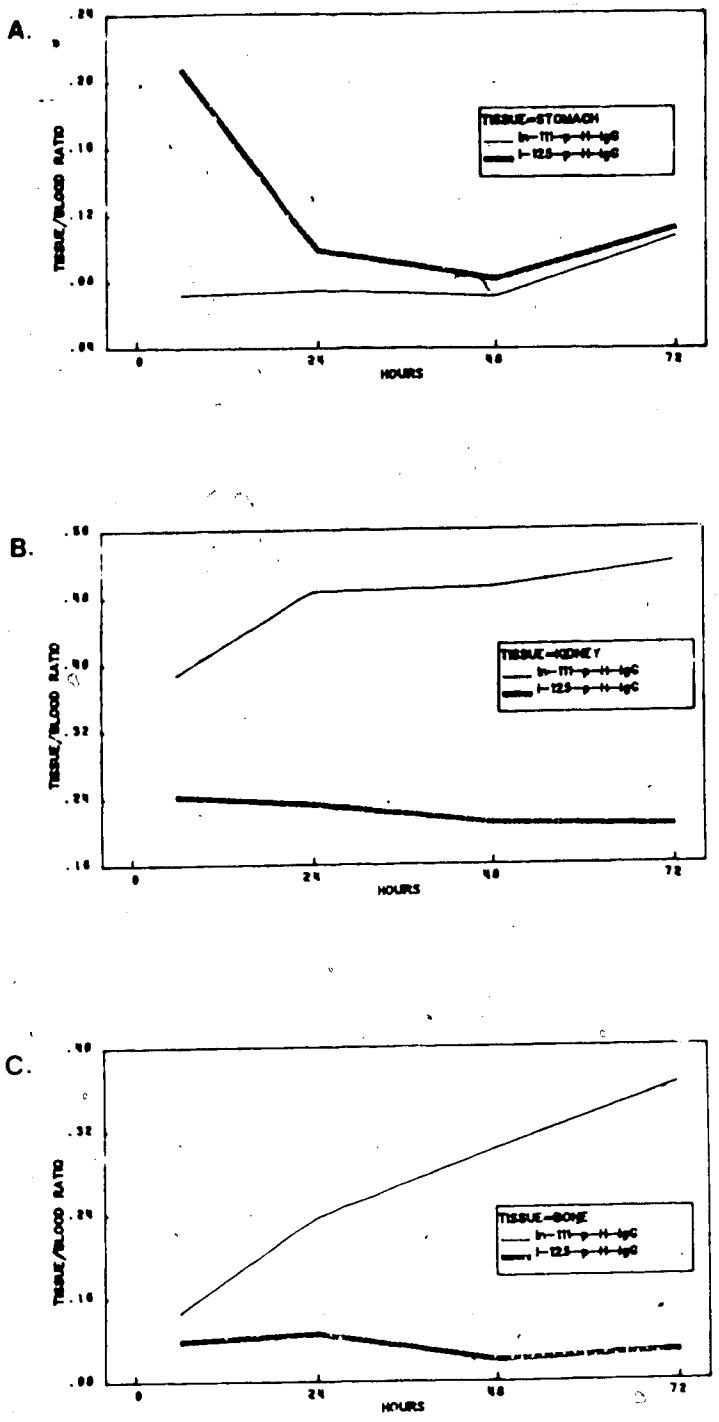


Figure 15b. Tissue to blood ratios for A) stomach, B) kidney and C) bone for ^{111}In-B-EDTA-poly-H-IgG and ^{125}I-poly-H-IgG. All points the mean of 5 animals with s.d. less than 20%.

Overall, the blood clearance of both isotopes is similar with ^{125}I being slightly higher than ^{111}In. The two organs that show major differences are the liver and kidney. High levels of ^{111}In in the liver from chelate labeled proteins have been reported by others[90,94] and range from 10 to 21% of the injected dose per gram of liver. The ^{111}In accumulation seen here could be due to the natural distribution of this preparation to the mouse liver or could be due to aggregates formed either *in vitro* from the labeling process or *in vivo* by complexation to circulating mouse proteins. This activity remains relatively constant over 72 hours. Kidney activity could be due to metabolism of the chelate complex off the protein by the liver and excretion through the kidneys or due to uptake of immune complexes by the kidney. The presence of iodine in the thyroid and stomach indicates that the iodinated protein is metabolized and the iodine released to the circulation. Bone activity shows accumulation of a small amount of ^{111}In over 72 hours also indicating the metabolism of the chelate labeled protein.

Analysis of percent dose remaining in the animals (see Appendix 1) shows higher retention of the ^{111}In as compared to the ^{125}I which is expected due to the high levels of ^{111}In in the liver.

3.1.9.2 DISTRIBUTION IN TUMOUR BEARING MICE

COMPARISON OF ^{111}In-B-EDTA-MAb-155H.7 TO ^{125}I-POLY-H-IGG AND ^{131}I-MAb-155H.7

Using a triple label experiment, iodinated poly-H-IgG and iodinated MAb-155H.7 were compared to ^{111}In-B-EDTA-MAb-155H.7. It is important to emphasize at this point that the poly-H-IgG used in these distribution studies is not an absolute control for the distribution of the radiolabeled MAbs. The proper control protein was not available at the time of these studies. Although it is not a monoclonal, subclass matched preparation, this compound acts as a methodology control and being a nonspecific protein gives some indication of the distribution of nonspecific immunoglobulins in the murine tumour model used here. Comparison of the percent of injected dose per gram of tissue data for ^{125}I-poly-H-IgG in normal Balb/c mice (see Appendix 1) with that generated in the TA3/Ha tumour bearing mice shows no significant difference between the two sets of data. Figures 16a and 16b present the results for all three proteins for selected organs over the time period studied. Appendix 1 summarizes percent of injected dose per gram of tissue and per organ data for this experiment.

The blood clearance of the poly-H-IgG in the tumour bearing animals is similar to that obtained in the normal Balb/c mouse distribution. The activity remains elevated with a slow decline over the 72 hour study period. The radioiodinated MAb-155H.7 and ^{111}In-chelated-MAb-155H.7 show definite differences in their distribution. The radioiodinated MAb-155H.7 follows the same general pattern as the poly-H-IgG with blood activity being slightly lower. The ^{111}In-B-EDTA-MAb-155H.7, however, shows rapid clearance from the blood with a steady decline over 72 hours.

Uptake of both ^{125}I and ^{131}I in thyroid and stomach indicate the metabolism of both the radioiodinated proteins. It should be noted that there is more of the ^{131}I in both of these organs suggesting that the MAb-155H.7 is metabolized more quickly

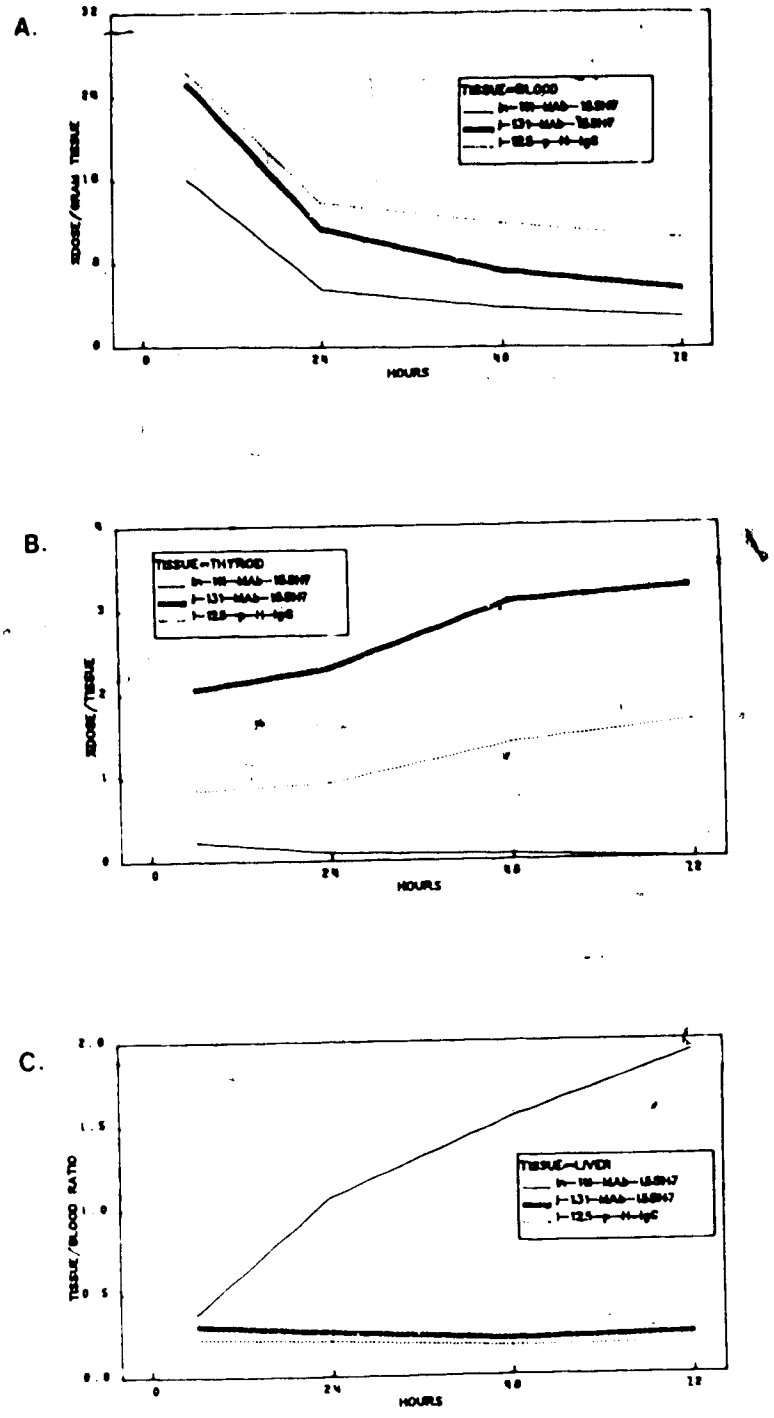


Figure 16a. A) Percent of injected dose per gram of blood, B) percent of injected dose per total thyroid and C) liver to blood ratio for ^{111}In-B-EDTA-MAb-155H.7, ^{131}I-MAb-155H.7 and ^{125}I-poly-H-IgG. All points the mean of 5 animals with s.d. less than 15%.

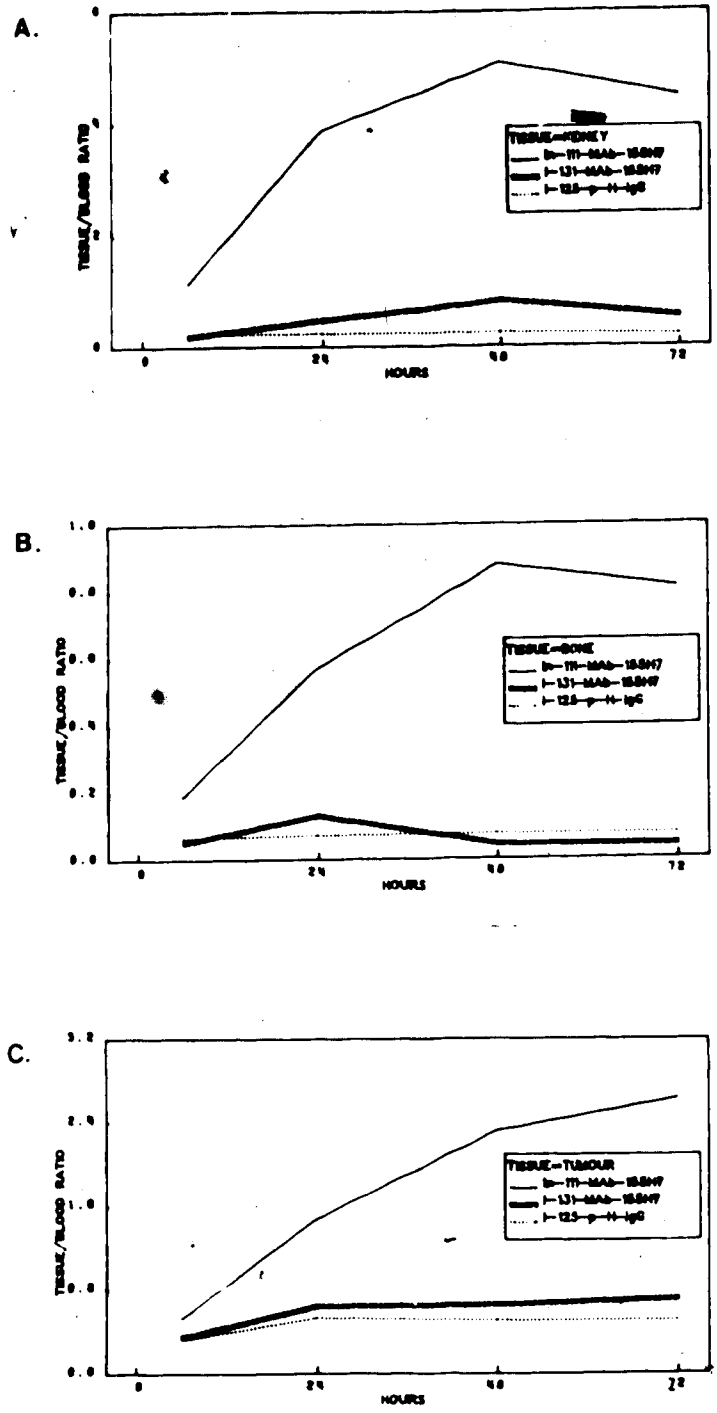


Figure 16b. Tissue to blood ratios for A) kidney, B) bone and C) tumour for ^{111}In-B-EDTA-MAb-155H.7, ^{125}I-MAb-155H.7 and ^{125}I-poly-H-IgG. All points the mean of 5 animals with s.d. less than 15%.

than the poly-H-IgG preparation.

Liver activity of ^{111}In is higher than either ^{131}I or ^{125}I. However, the ^{111}In levels in the tumour bearing mice are not nearly as high as were seen in the normal Balb/c mice with the chelate labeled poly-H-IgG (6% of the injected dose per gram of liver for ^{111}In-B-EDTA-MAb-155H.7 as compared to 18% for ^{111}In-B-EDTA-poly-H-IgG at 72 hours - see Appendix 1). The radioiodinated proteins also show a difference with ^{131}I-MAb-155H.7 being higher in the liver than ^{125}I-poly-H- IgG.

The kidney shows a significant difference in the amount of ^{111}In and ^{131}I retained by this organ. Since PAB-EDTA-^{111}In is excreted quickly through the kidney, part of the high uptake seen here could be due to the excretion of metabolized chelate. Another possibility that could explain the high uptake in the kidney is active uptake of the MAb itself through specific receptors. Boniface *et al.*[116] have demonstrated the presence of a T-like structure in the kidney that binds peanut lectin. Analysis of the ratio of the kidney to blood ratio for ^{111}In over the kidney to blood ratio for ^{131}I shows a constant of 7.22 ± 0.93. The stability of this ratio over 72 hours points to the persistence of the MAb in the kidney and may be support for the active uptake of the MAb by the kidneys.

Bone uptake of ^{111}In is low when compared to that seen in the normal mouse distribution and indicates the high stability of this chelate *in vivo* with regard to transferrin.

Tumour uptake is virtually the same for the radioiodinated proteins but ^{111}In accumulates over 72 hours. The lack of difference between radioiodinated poly-H-IgG and radioiodinated MAb-155H.7 was surprising. Although the MAb-155H.7 was not generated against the TA3/Ha tumour cell, it was hoped that some specific accumulation of the MAb would occur *in vivo* due to the large concentration of epiglycanin carrying the T-antigen structure on the surface of the cell. This appears not to be the case with the radioiodinated MAb-155H.7. The

accumulation of both radioiodinated proteins in the tumour could be due to non-specific processes or there may be radioiodinated antibodies in the poly-H-IgG preparation that may have some specificity for the TA3/Ha cells. Since this is a polyclonal preparation, it is possible that there may be a variety of antibodies present that could bind to the tumour cells. Evidence is now appearing in the literature that suggests that local metabolic processes in the tumour cells may be releasing radioiodine from radioiodinated proteins and returning it to the circulation[93,94] whereas the catabolized ^{111}In remains stored in the tumour cell. This is also supported by these studies where there is steady accumulation of the ^{111}In over 72 hours and a stable or steady decrease of radioiodine levels in the tumour over 72 hours. Tissue analysis of the animals used in the scintigraphic studies (data not shown) supports the active accumulation of ^{111}In from the chelated MAb compared to ^{111}In from ^{111}In-citrate. After 96 hours, the percent dose per gram of tumour for ^{111}In from the chelated MAb was 9.6 whereas that for ^{111}In from ^{111}In-citrate was 3.6.

Examining the percent dose remaining in each animal (see Appendix 1) shows highest retention of ^{111}In with the ^{125}I from poly-H-IgG only slightly lower. Retention of ^{131}I from MAb-155H.7 is very low and declines rapidly from 6 to 24 hours with a slow but steady decrease thereafter.

From these studies it is evident that the radioiodine label for MAb distribution is less stable than ^{111}In. As well, a distinct difference is observed in the general distribution of the two labels. The rapid blood clearance of ^{111}In labeled MAb enhances the tumour to blood ratio by reducing the blood background normally seen with radioiodinated MAbs. Instability of the radioiodine label is evidenced by uptake in the stomach and thyroid and little or no accumulation in the tumour.

3.1.9.3 WHOLE BODY SCINTIGRAPHIC STUDIES IN TUMOUR-BEARING MICE

Whole body scanning was performed on mice bearing the TA3/Ha tumour subcutaneously on the lower right flank. Mice were injected with single preparations of either ^{111}In-B-EDTA-MAb-155H.7, ^{111}In-citrate, ^{131}I-MAb-155H.7 or ^{131}I-poly-H-IgG.

Figures 17 and 18 summarize the 24, 48, 72 and 96 hour scans performed on these mice.

The distribution pattern seen in the scans is identical to that observed in the biodistribution study involving these three agents. Rapid blood clearance of the ^{111}In-B-EDTA-MAb-155H.7 and ^{111}In- citrate is observed as early as 24 hours. Kidney and tumour activity are visible for the ^{111}In-B-EDTA-MAb-155H.7 with only kidney showing for the ^{111}In-citrate. Progressive tumour accumulation is demonstrated for the ^{111}In labeled MAb with the ^{111}In-citrate largely representing tumour blood pool. The radioiodinated MAb-155H.7 and poly-H-IgG demonstrate the rapid de-iodination observed in the biodistribution study with accumulation of activity in the thyroid and abdominal (stomach) area. The tumour is clearly visible with both radioiodinated proteins however analysis of the animals by dissection and counting after 96 hours shows tumour to blood ratios for ^{111}In of 9 : 1 and less than 1 : 1 for both the radioiodinated proteins and ^{111}In-citrate. If the mice had had their thyroids and stomachs 'blocked' (by preadministration of KI in their drinking water), the kidneys of these animals would also have been visible further obscuring the abdominal region.

These scans along with the supporting biodistribution data point to the superiority of the ^{111}In labeled MAb over the radioiodinated preparation. Progressive tumour accumulation and rapid blood clearance enhance the tumour imaging properties of the MAb. As well, ^{111}In is a superior imaging radioisotope due to its favourable emission characteristics as compared to ^{131}I, the iodine radioisotope most often used for imaging studies. Rapid elimination of the chelated ^{111}In reduces the risk of exposure to the patient thereby foregoing the procedures involved in preparing the patient for radioiodine imaging.

At the present time, ^{131}I-MAb-155H.7 is undergoing Phase I (toxicity) clinical trials at the Cross Cancer Institute for the *in vivo* detection of ovarian and colorectal carcinomas. These studies were initiated prior to completion of the presented here and future studies will most likely incorporate the use of ^{111}In for clinical investigation.

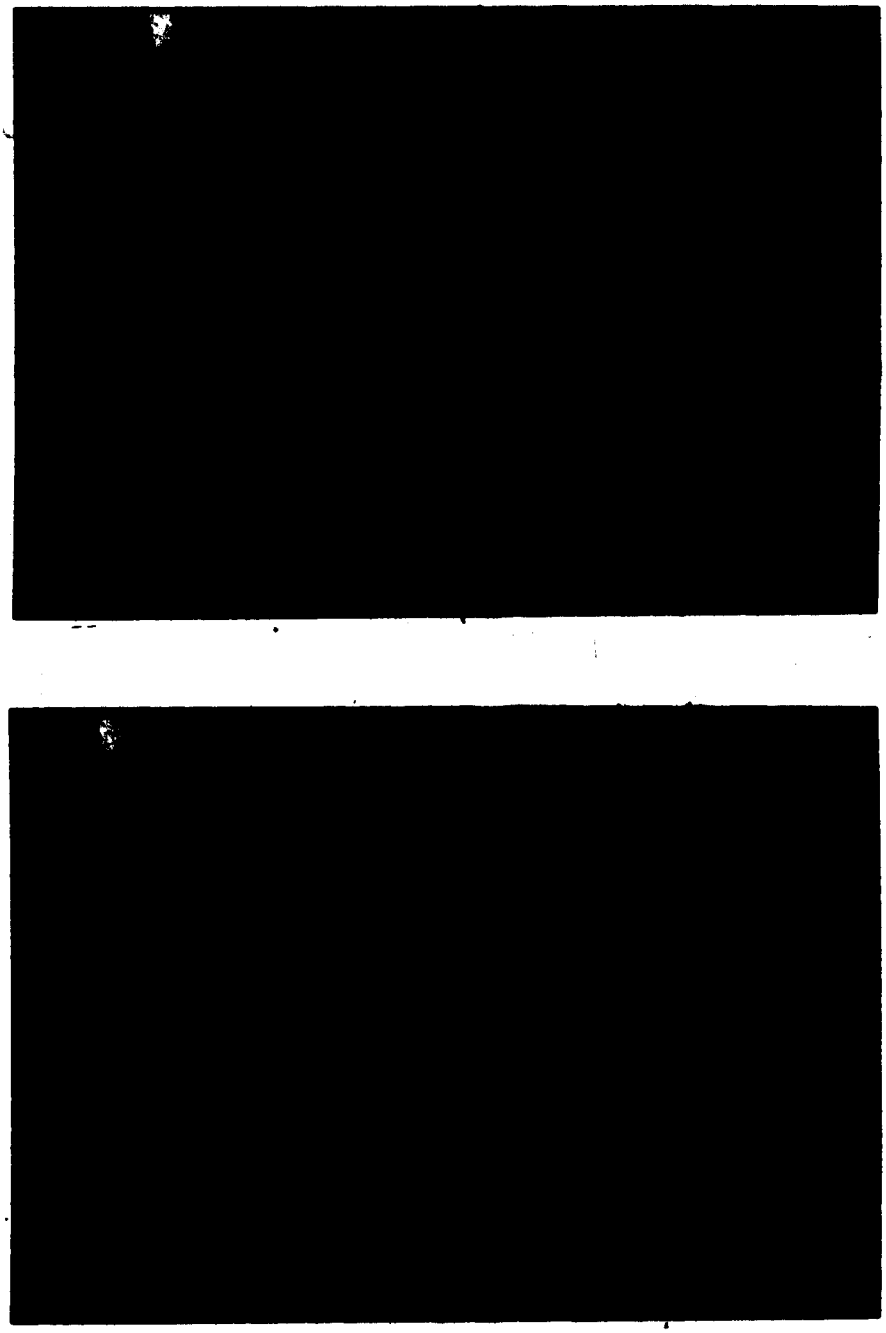


Figure 17. Twenty four and 48 hour scans of TA3/Ha tumour bearing mice injected I.V. with ^{111}In-citrate, ^{111}In-B-EDTA-MAb-155H.7, ^{131}I-poly-H-IgG or ^{131}I-MAb-155H.7. Tumours were implanted subcutaneously on the lower right flank; all images are posterior views.

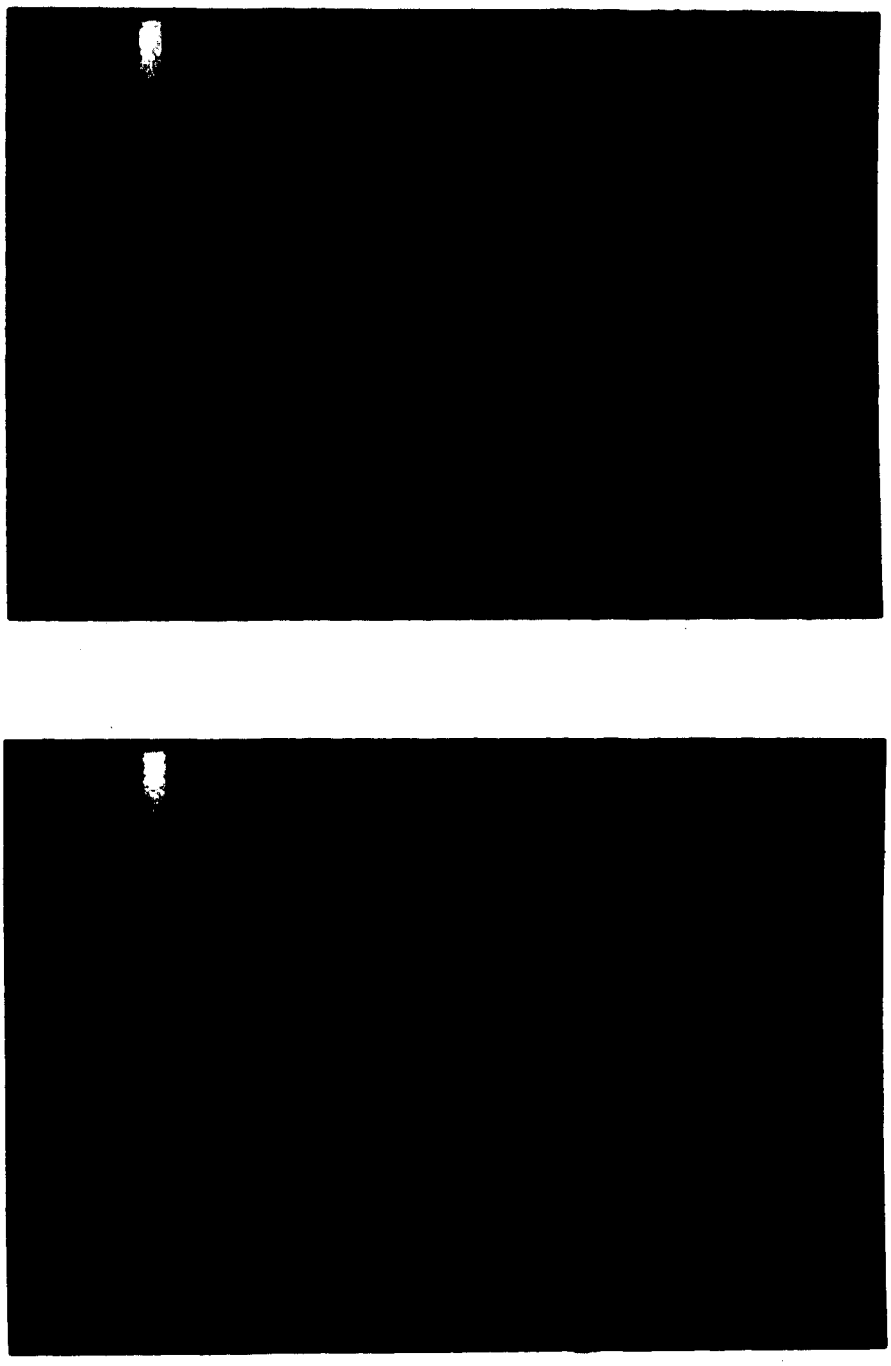


Figure 18. Seventy two and 96 hour scans of TA3/Ha tumour bearing mice injected I.V. with ^{111}In-citrate, ^{111}In-B-EDTA-MAb-155H.7, ^{131}I-poly-H-IgG or ^{131}I-MAb-155H.7. Tumours were implanted subcutaneously on the lower right flank; all images are posterior views.

4. SUMMARY AND CONCLUSIONS

The aims of the work presented here can be summarized as follows: 1) to determine if the derivatized bifunctional chelating agent, PAB-EDTA, could be successfully used to label MAb with radiometal cations, such as ^{111}In and ^{67}Ga, and 2) to determine if the bifunctional chelate labeled MAb was superior to radioiodine labeled MAb with respect to *in vitro* MAb function and *in vivo* use as a diagnostic tool for cancer imaging.

The bifunctional chelate was shown to successfully bind radioindium and radiogallium in the unconjugated state and the radioindium chelate was stable against transchelation to transferrin *in vitro*. The bromoacetamido- derivative was superior to the diazonium salt derivative for labeling MAbs and radioindium was the preferred radioisotope as reduced labeling yields with radiogallium were obtained. This was due mainly to the presence of non-radioactive metal ions discovered in the radiogallium preparations by INAA. Comparative *in vitro* testing of BrAc-B-EDTA labeled MAb-155H.7 and radioiodinated MAb-155H.7 indicated that the bifunctional chelate-labeled MAb-155H.7 was only marginally superior to the radioiodinated species. The advantages of using the bifunctional chelate, such as, advance labeling and long term storage of the bifunctional chelate labeled MAb with addition of the radioisotope as the final step, promote the use of this reagent although the *in vitro* effects of the labeling technique were not much different from radioiodination.

As well, the importance of quality control of both bifunctional chelate labeled and radioiodinated MAbs was emphasized by the results obtained with the various *in vitro* assays used to assess MAb function.

The *in vivo* testing demonstrated two pertinent points relevant to the use of bifunctional chelates for labeling MAb. The preliminary biodistribution of the ^{125}I-PNB-EDTA-^{111}In in normal mice demonstrated the *in vivo* stability of the chelated ^{111}In. Although the iodine label was relatively labile, as evidenced by increased amounts in thyroid, liver and stomach compared to ^{111}In, the two labels indicate the rapid excretion of the chelate by the kidneys. This rapid excretion is characteristic of radiometal chelates of EDTA and DTPA and less than 2% of the injected dose is remaining in the animals at 24 hours. The lack

of bone and liver uptake lends support to the fact that there is no transchelation of ^{111}In to transferrin over the time period studied. This compares favourably to the results obtained by Yeh *et al.*[31] where they demonstrated that derivatized PAB-EDTA labeled proteins lost only 0.11% of their ^{111}In to transferrin per day.

The difference in biodistribution of ^{111}In-B-EDTA-MAb-155H.7 and ^{131}I-MAb-155H.7 was also shown in TA3/Ha tumour bearing mice. The blood clearance of ^{111}In-B-EDTA-MAb-155H.7 was faster than either ^{131}I-MAb-155H.7 or ^{125}I-poly-H-IgG. As well, the ^{131}I-MAb-155H.7 cleared faster than ^{125}I-poly-H-IgG. Extensive de-iodination of the ^{131}I-MAb-155H.7 was demonstrated by thyroid and stomach uptake of the tracer and from analysis of the percent of injected dose remaining data. Low tumour uptake was observed with ^{131}I-MAb-155H.7 and the tumour to blood ratios were not significantly different from the ^{125}I-poly-H-IgG used as the control protein. The ^{111}In-B-EDTA-MAb-155H.7, however, showed progressive accumulation of ^{111}In in the tumour and a steady level of accumulation in the liver over 72 hours. As a result, the rapid blood clearance showed enhanced tumour to blood ratios for ^{111}In. The significant levels in the kidney of ^{111}In may be related to specific uptake by the kidneys of the radiolabeled MAb combined with metabolically released ^{111}In-chelate. The different fates of radioiodine and radioindium labeled MAbs is supported by other researchers as well.[42,59,94] Dehalogenation of the radioiodinated MAb has been shown to occur not only in the liver but also at the level of the tumour cell itself. Catabolized ^{111}In, on the other hand, undergoes a different fate with either rapid elimination through the kidneys or sequestration in the tumour cell by mechanisms as yet undefined.

The labeling of MAbs with derivatized PAB-EDTA has been shown to be a feasible, reproducible method to attach radioisotopes other than radioiodine to MAbs and has also demonstrated its superiority to radioiodinated MAb *in vivo*.

REFERENCES

1. Hericourt J, Richet C. Traitement d'un cas de sarcome par la serotherapie. *C.R. Hebd. Seances Acad. Sci.* 120:948 1895.

2. Hericourt J, Richet C. De la serotherapie dans la traitement du cancer. *C.R. Hebd. Seances Acad. Sci.* 121:567 1895.

3. Pressman D, Korngold L. The *in vivo* localization of anti-Wagner osteogenic sarcoma antibodies. *Cancer* 6:619 1953.

4. Korngold L, Pressman D. The localization of antilymphosarcoma antibodies in the Murphy lymphosarcoma of the rat. *Cancer Res.* 14:96 1954.

5. Pressman D, Day ED, Blan M. The use of paired labeling in the determination of tumor-localizing antibodies. *Cancer Res.* 17:845 1957.

6. Pressman D, Pressman R. Computer programs for paired and triad radioiodine label techniques in radioimmunochemistry. *Int. J. Appl. Radiat. Isot.* 18:617 1965.

7. Bale WF, Spar IL, Goodland RL. Experimental radiation therapy of tumors with ^{131}I-carrying antibodies to fibrin. *Cancer Res.* 20:1488 1960.

8. Hellström KE, Brown JP, Hellström I. Monoclonal antibodies to tumor antigens. *Contemp. Top. Immunobiol.* 11:117 1980.

9. von Kleist S. News and views on tumor markers: the use of radioactive antibodies against cell-bound antigens. *Nuklearmedizin* 23:1 1984.

10. Hellström KE, Hellström I, Brown JP. Human tumor-associated antigens identified by monoclonal antibodies. *Springer Semin. Immunopathol.* 5:127 1982.

11. Larson SM, Carrasquillo JA, Reynolds JC. Radioimmunodetection and radioimmunotherapy. *Cancer Invest.* 2:363 1984.

12. Springer GF. T and Tn, general carcinoma autoantigens. *Science* 224:1198 1984.

13. Reif AE, Curtis LE, Duffield R, *et al.* Trial of radiolabeled antibody localization in metastases of a patient with a tumour containing carcinoembryonic antigen (CEA). *J. Surg. Oncol.* 6:133 1974.

14. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256:495 1975.

15. Belliveau RE. Transport of radiolabeled antibodies, in 'Biological Transport of Radiotracers', LG Colombetti, ed. CRC Press, Inc. Boca Raton, Fla. 1982. pp 245-260.

16. Parr B. Lymphocyte hybridization: aspects of the technology, in 'Immunoassays', CA Bizollon, ed. Elsevier Science Pub. Amsterdam. 1984. pp 25-33.

17. Phillips AP, Martin KH, Horton WH. The choice of methods for immunoglobulin IgG purification: yield and purity of antibody activity. *J. Immunol. Meth.* 74:385 1984.

18. Stanker LH, Vanderlaan M, Juarez-Salinas H. One-step purification of mouse monoclonal antibodies from ascites fluid by hydroxylapatite - chromatography. *J. Immunol. Meth.* 76:157 1985.

19. Brooks TL, Stevens A. Preparative HPLC purification of IgG and IgM monoclonal antibodies. *American Laboratory* Oct:1985.

20. Lane HC, Fauci AS. Establishment of human-human and human-mouse B cell hybrids and their use in the study of B cell activation, in 'Monoclonal Antibodies: Probes for the Study of Autoimmunity and Immunodeficiency', Haynes BF, Eisenbarth GS, eds: Acad. Press Inc. Orlando, Fla. 1983. pp 131-151.

21. Regoeczi E. Iodine-labeled plasma proteins. Vol. I CRC Press Inc. Boca Raton, Fla. 1984.

22. Lederer CM, Shirley VS, eds. Table of Isotopes. 7th ed. John Wiley and Sons. Toronto. 1978.

23. McFarlane AS. Efficient trace labelling of proteins with iodine. *Nature* 182:53 1958.

24. Hunter WM, Greenwood FC. Preparation of iodine-131 labeled human growth hormone of high specific activity. *Nature* 194:495 1962.

25. Fraker PJ, Speck JC, Jr. Protein and cell membrane iodination with a sparingly soluble chloroamide 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril. *Biochem. Biophys. Res. Commun.* 80:849 1978.

26. Marchalonis JJ. An enzymic method for trace iodination of immunoglobulins and other proteins. *Biochem. J.* 113:299 1969.

27. Pommier J, Solokoff L, Nunez J. Enzymatic iodination of protein. Kinetics of iodine formation and protein iodination catalyzed by horseradish peroxidase. *Eur. J. Biochem.* 38:497 1973.

28. Dubin A, Silberring J. Catalytic iodination of proteins by horse myeloperoxidase in solid state. *Anal. Biochem.* 72:372 1976.

29. Bolton AE, Hunter WM. The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. *Biochem. J.* 133:529 1973.

30. Pennisi F, Rosa U. Preparation of radioiodinated insulin by constant current electrolysis. *J. Nucl. Med. Biol.* 13:64 1969.

31. Benisek WF, Richards FM. Attachment of metal-chelating functional groups to hen egg white lysozyme - an approach to introducing heavy atoms into protein crystals. *J. Biol. Chem.* 243:4267 1968.

32. Pritchard JH, Ackerman M, Tubis M, *et al.* Indium-111-labeled antibody heavy metal chelate conjugates: a potential alternative to radioiodination. *Proc. Soc. Exp. Biol. Med.* 151:297 1976.

33. Gokce A, Nakamura RM, Tubis M, *et al.* Synthesis of indium-labeled antibody-chelate conjugate for radioassays. *Int. J. Nucl. Med. Biol.* 9:85 1982.

34. Yokoyama A, Ohmomo Y, Horiuchi K, *et al.* Deferoxamine, a promising bifunctional chelating agent for labeling proteins with gallium: Ga-67 DF-HSA: concise communication. *J. Nucl. Med.* 23:909 1982.

35. Janoki GA, Harwig JF, Chanachai W, *et al.* [^{67}Ga] desferrioxamine-HSA: synthesis of chelon protein conjugates using carbodiimide as a coupling agent. *Int. J. Appl. Radiat. Isot.* 34:871 1983.

36. Eckelman WC, Karesh SM, Reba RC. New compounds: fatty acid and long chain hydrocarbon derivatives containing a strong chelating agent. *J. Pharm. Sci.* 64:704 1975.

37. Hnatowich DJ, Layne WW, Childs RL. The preparation and labeling of DTPA-coupled albumin. *Int. J. Appl. Radiat. Isot.* 33:327 1982.

38. Hnatowich DJ, Layne WW, Childs RL, *et al.* Radioactive labeling of antibody: a simple and efficient method. *Science* 220:613 1983.

39. Krecjarek GE, Tucker KL. Covalent attachment of chelating groups to macromolecules. *Biochem. Biophys. Res. Commun.* 77:581 1977.

40. Khaw BA, Strauss HW, Cahill SL, *et al.* Sequential imaging of indium-111-labeled monoclonal antibody in human mammary tumors hosted in nude mice. *J. Nucl. Med.* 25:592 1984.

41. Zoghbi SS, Neumann RD, Gottschalk A. A modified procedure for rapid labelling of low concentrations of bioactive proteins with indium-111. *Int. J. Nucl. Med. Biol.* 12:159 1985.

42. Hagan PL, Halpern SE, Chen A, *et al. In vivo* kinetics of radiolabeled monoclonal anti-CEA antibodies in animal models. *J. Nucl. Med.* 26:1418 1985.

43. Wang TST, Srivastava SC, Fawwaz RA, *et al.* A comparison of the cyclic anhydride and mixed anhydride methods for ^{111}In-DTPA chelation to monoclonal antibodies. *Nuklearmedizin* 4:193 1984.

44. Paik CH, Murphy PR, Eckelman WC, *et al.* Optimization of the DTPA mixed anhydride reaction with antibodies at low concentration. *J. Nucl. Med.* 24:932 1983.

45. Means GE, Feeney RE. Chemical modification of proteins. Holden Day. San Franscisco, Calif. 1971. pp 68-138.

46. Chanachai W, Shani J, Wolf W, *et al.* Standardization of CDI-mediated DTPA-coupling to IgG and IgG_{2a} antibodies for ^{113m}In labeling. *Int. J. Nucl. Med. Biol.* 12:289 1985.

47. Paxton RJ, Jakowatz JG, Beatty JD, *et al.* High specific activity ^{111}In-labeled anticarcinoembryonic antigen monoclonal antibody: improved method for the synthesis of diethylenetriaminepentaacetic acid conjugates. *Cancer Res.* 45:5694 1985.

48. Najafi A, Hutchinson N. The use of DTPA diactivated ester in coupling DTPA to proteins. *Appl. Radiat. Isot.* 37:548, 1986.

49. Goodwin DA, Sundberg MW, Diamanti CI, *et al.* Indium-111 radiopharmaceuticals in cancer localization, in 'Radiologic and other Biophysical Methods of Tumour Diagnosis' 18th Annual Conference on Cancer (Proceedings). Yearbook Med. Pub. Inc. Chicago. 1973. pp 57-88.

50. Sundberg MW, Meares CF, Goodwin DA, *et al.* Chelating agents for the binding of metal ions to macromolecules. *Nature* 250:587 1974.

51. Sundberg MW, Meares CF, Goodwin DA, *et al.* Selective binding of metal ions to macromolecules using bifunctional analogs of EDTA. *J. Med. Chem.* 17:1304 1974.

52. Meares CF, Goodwin DA, Leung CS-H, *et al.* Covalent attachment of metal chelates to proteins: the stability *in vivo* and *in vitro* of the conjugate of albumin with a chelate of indium-111. *Proc. Natl. Acad. Sci. (USA)* 73:3803 1976.

53. Yeh SM, Meares CF, Goodwin DA. Decomposition rates of radiopharmaceutical indium chelates in serum. *J. Radioanal. Chem.* 53:327 1979.

54. Leung CS-H, Meares CF, Goodwin DA. The attachment of metal chelating groups to proteins: tagging of albumin by diazonium coupling and use of the products as radiopharmaceuticals. *Int. J. Appl. Radiat. Isot.* 29:687 1978.

55. Wensel TG, Meares CF. "Bifunctional" chelating agents for binding metal ions to proteins, in 'Radioimmunoimaging and Radioimmunotherapy', Burchiel SW, Rhodes BA, eds. Elsevier Science Pub. Co., Inc. New York. 1983. pp 185-196.

56. Yeh SM, Sherman DG, Meares CF. A new route to "bifunctional" chelating agents: conversion of amino acids to analogs of ethylenediaminetetraacetic acid. *Anal. Biochem.* 100:152 1979.

57. Meares CF, DeReimer LH, Leung CS-H, *et al.* Properties *in vivo* of chelate tagged proteins and polypeptides, in 'Modification of Proteins: Food, Nutritional and Pharmacological Aspects', Feeney RE, Whitaker JR, eds. Am. Chem. Soc. Washington, D.C. 1982. pp 369-387.

58. Meares CF, McCall MJ, Reardon DT, *et al.* Conjugation of antibodies with bifuncitional chelating agents: isothiocyanate and bromoacetamide reagents, methods of analysis and subsequent addition of metal ions. *Anal. Biochem.* 142:68 1984.

59. Goodwin DA, Meares CF, DeReimer LH, *et al.* Clinical studies with In-111 BLEDTA, a tumor-imaging conjugate of bleomycin with a bifunctional chelating agent. *J. Nucl. Med.* 22:787 1981.

60. Goodwin DA, Meares CF, McCall MJ, *et al.* Chelate conjugates of monoclonal antibodies for imaging lymphoid structures in the mouse. *J. Nucl. Med.* 26:493 1983.

61. Paik CH, Herman DE, Eckelman WC, *et al.* Synthesis, plasma clearance and *in vitro* stability of protein containing a conjugated indium-111 chelate. *J. Radioanal. Chem.* 57:553 1980.

62. Moi MK, Meares CF, McCall MJ, *et al.* Copper chelates as probes of biological systems: stable copper complexes with a macrocyclic bifunctional chelating agent. *Anal. Biochem.* 148:249 1985.

63. Welch MJ, Moerlein S. Radiolabeled compounds of biomedical interest containing radioisotopes of gallium and indium, in 'Inorganic Chemistry in Biology and Medicine', Martell AE, ed. Am. Chem. Soc., Washington, D.C. 1980. pp 121-140.

64. Wagner SJ, Welch MJ. Gallium-68 labelling of albumin and albumin microspheres. *J. Nucl. Med.* 20:428 1979.

65. Maziere B, Loc'h C, Ricordel Y, *et al.* Regional cerebral blood pool measurements using ^{113}Sn-labeled proteins. Proceedings, 3rd World Congress of Nuclear Medicine and Biology. Paris. 1982. pp 666-669.

66. Fritzberg AR. Radiopharmaceuticals: Progress and Clinical Perspectives. Vol I and II. CRC Press, Inc. Boca Raton, Fla. 1986.

67. Rhodes BA, Burchiel SW. Radiolabeling of antibodies with technetium-99m, in 'Radioimmunoimaging and Radioimmunotherapy', Rhodes BA, Burchiel SW, eds. Elsevier Science Pub. Co., Inc. New York. 1983. pp 207-222.

68. Paik CH, Phan LNB, Hong JJ, *et al.* The labeling of high affinity sites of antibodies with ^{99m}Tc. *Int. J. Nucl. Med. Biol.* 12:3 1985.

69. Buschbaum D, Randall B, Hanna D, *et al.* Comparison of the distribution and binding of monoclonal antibodies labeled with 131-iodine or 111-indium. *Eur. J. Nucl. Med.* 10:398 1985.

70. Buchegger F, Haskell CM, Schreyer M, *et al.* Radiolabeled fragments of monoclonal antibodies against carcinoembryonic antigen for localization of human colon carcinoma grafted to nude mice. *J. Exp. Med.* 158:413 1983.

71. Buschbaum DJ, Hanna DE, Randall BC, *et al.* Radiolabeling of monoclonal antibody against carcinoembryonic antigen with ^{90}Y and biodistribution studies. *Int. J. Nucl. Med. Biol.* 12:79 1985.

72. Haskell CM, Buchegger F, Schreyer M, *et al.* Monoclonal antibodies to carcinoembryonic antigen: ionic strength as a factor in the selection of [illegible] for immunoscintigraphy. *Cancer Res.* 43:3857 1983.

73. Hagan PL, Halpern SE, Dillman RO, *et al.* Tumor [illegible] effect on monoclonal antibody uptake in tumor models. *J. Nucl. Med.* 27:422 1986.

74. Hagan PL, Halpern SE, Chen A, *et al. In vivo* kinetics of radiolabeled monoclonal anti-CEA antibodies in animal models. *J. Nucl. Med.* 26:1418 1985.

75. Martin KW, Halpern SE. Carcinoembryonic antigen production, secretion, and kinetics in BALB/c mice and a nude mouse-human tumor model. *Cancer Res.* 44:5478 1984.

76. Halpern SE, Hagan PL, Garver PR, *et al.* Stability, characterization, and kinetics of ^{111}In-labeled monoclonal antitumor antibodies in normal animals and nude mouse-human tumor models. *Cancer Res.* 43:5347 1983.

77. Wahl RL, Parker CW, Philpott GW. Improved radioimaging and tumor localization with monoclonal F(ab')$_2$. *J. Nucl. Med.* 24:316 1983.

78. Jakowatz JG, Beatty BG, Vlahos WG, *et al.* High-specific activity ^{111}In-labeled anticarcinoembryonic antigen monoclonal antibody: biodistribution and imaging in nude mice bearing human colon cancer xenografts. *Cancer Res.* 45:5700 1985.

79. Philben VJ, Jakowatz JG, Beatty BG, *et al.* The effect of tumor CEA content and tumor size on tissue uptake of indium-111 labeled anti-CEA monoclonal antibody. *Cancer* 57:571 1986.

80. Epenetos AA, Nemmon CC, Arklie J, *et al.* Detection of human cancer in an animal model using radio-labelled tumour-associated monoclonal antibodies. *Br. J. Cancer* 46:1 1982.

81. Pimm MV, Perkins AC, Baldwin RW. Simultaneous localization of two monoclonal antibodies in a human carcinoma xenograft. *IRCS J. Med. Sci.* 13:499 1985.

82. Pimm MV, Embleton MJ, Perkins AC, *et al. In vivo* localization of anti-osteogenic sarcoma 791T monoclonal antibody in osteogenic sarcoma xenografts. *Int. J. Cancer* 30:75 1982.

83. Doiullard J-Y, Chatal J-F, Saccavini JC, *et al.* Pharmacokinetic study of radiolabeled anti-colorectal carcinoma monoclonal antibodies in tumour-bearing nude mice. *Eur. J. Nucl. Med.* 11:107 1985.

84. Herlyn D, Powe J, Alavi A, *et al.* Radioimmunodetection of human tumour xenografts by monoclonal antibodies. *Cancer Res.* 43:2731 1983.

85. Hedin A, Wahren B, Hammarstrom S. Tumor localization of CEA-containing human tumors in nude mice by means of monoclonal anti-CEA antibodies. *Int. J. Cancer* 30:547 1982.

86. Warenius HM, Galfre D, Bleehan NM, *et al.* Attempted targeting of a monoclonal antibody in a human tumour xenograft system. *Eur. J. Cancer Clin. Oncol.* 17:1009 1981.

87. Beaumier PL, Krohn KA, Carrasquillo JA, *et al.* Melanoma localization in nude mice with monoclonal Fab against p97. *J. Nucl. Med.* 26:1172 1985.

88. Matzku S, Hellström I. Tumor localization with F(ab')$_2$ fragments of monoclonal antibody 96.5 specific for the p97 marker of human melanoma. Proceedings of the 3rd World Congress on Nuclear Medicine and Biology. Paris. 1982. pp 3659-3661.

89. Hwang KM, Keenan AM, Frincke; *et al.* Dynamic interaction of ^{111}indium-labeled monoclonal antibodies with surface antigens of solid tumors visualized *in vivo* by external scintigraphy. *J. Natl. Cancer Inst.* 76:849 1986.

90. Bumol TF, Reisfeld RA. Unique glycoprotein-proteoglycan complex defined by monoclonal antibody on human melanoma cells. *Proc. Natl. Acad. Sci. (USA)* 79:1245 1982.

91. Fawwaz RA, Wang TST, Estabrook AE, *et al.* Immunoreactivity and biodistribution of indium-111-labeled monoclonal antibody to a human high molecular weight-melanoma associated antigen. *J. Nucl. Med.* 26:488 1985.

92. Gomibuchi M, Saxton RE, Lake RR, *et al.* Radioimmunodetection of human melanoma tumor xenografts with human monoclonal antibodies. *Nucl. Med. Biol.* 13:13 1986.

93. Stuhlmiller GM, Sullivan DC, Vervaert CE, *et al. In vivo* localization using tumor-specific monkey xenoantibody, alloantibody, and murine monoclonal xenoantibody. *Ann. Surg.* 194:592 1981.

94. Pimm MV, Perkins AC, Baldwin RW. Differences in tumour and normal tissue concentration of iodine- and indium-labelled monoclonal antibody. II. Biodistribution studies in mice with human tumour xenografts. *Eur. J. Nucl. Med.* 11:300 1985.

95. Perkins AC, Pimm MV, Birch MK. The preparation and characterization of ^{111}In-labeled 791T/36 monoclonal antibody for tumour immunoscintigraphy. *Eur. J. Nucl. Med.* 10:296 1985.

96. Moshakis V, McIlhinney RA, Raghavan D, *et al.* Localization of human tumour xenografts after i.v. administration of radiolabelled monoclonal antibodies. *Br. J. Cancer* 44:91 1981.

97. Moshakis V, McIlhinney RA, Neville AM. Cellular distribution of monoclonal antibody in human tumours after i.v. administration. *Br. J. Cancer* 44:663 1981.

98. McIlhinney RAJ, Raghavan D, Neville AM. Monoclonal antibodies to detect human tumours: an experimental approach. *J. Clin. Path.* 34:314 1981.

99. Zalutsky MR, Colcer D, Kaplan WD, *et al.* Radioiodinated B6.2 monoclonal antibody: further characterization of a potential radiopharmaceutical for the identification of breast tumors. *Int. J. Nucl. Med..Biol.* 12:227 1985.

100. Henkel RD, Kennedy RC, Sparrow JT, *et al. In vivo* detection of human hepatoma secreting hepatitis B surface antigen in nude mice with radiolabeled monoclonal antibodies that recognize distinct epitopes. *Clin. Immun. Immunopath.* 35:146 1985.

101. Brown JM, Greager JA, Pavel DG, *et al.* Localization of radiolabeled monoclonal antibody in a human soft tissue sarcoma xenograft. *J. Natl. Cancer Inst.* 75:637 1985.

102. Brown JM. Detection of a human sarcoma-associated antigen with monoclonal antibodies. *Cancer Res.* 43:2113 1983.

103. Epenetos AA, Shephard J, Brilton KE, *et al.* Radioimmunodiagnosis of ovarian cancer using ^{123}I-labelled, tumor associated monoclonal antibodies. *Cancer Det. Prev.* 7:45 1984.

104. Anderson WT, Strand M. Stability, targeting and biodistribution of scandium-46 and gallium-67-labeled monoclonal antibody in erythroleukemic mice. *Cancer Res.* 45:2154 1985.

105. Scheinberg DA, Strand M. Kinetic and catabolic considerations of monoclonal antibody targeting in erythroleukemic mice. *Cancer Res.* 43:265 1983.

106. Scheinberg DA, Strand M, Gansow OA. Tumor imaging with radioactive metal chelates conjugated to monoclonal antibodies. *Science* 215:1511 1982.

107. Stavrou D, Mellert W, Bilza T, *et al.* Localization of experimental glioma grafts by means of iodinated monoclonal antibodies and radionuclide imaging. *Acta. Neuropathol. (Berlin)* 66:340 1985.

108. Bernhard MI, Hwang KM, Foon KA, *et al.* Localization of ^{111}In- and ^{125}I-labeled monoclonal antibody in guinea pigs bearing Line 10 hepatocarcinoma tumors. *Cancer Res.* 43:4429 1983.

109. Mew D, Alcorn L, Lyster D, *et al.* Imaging studies with Tc-99m-labeled tumor specific monoclonal antibodies to a transplantable murine tumor, in 'Radioimmunoimaging and Radioimmunotherapy', Burchiel SW, Rhodes BA, eds. Elsevier Science Pub, Co. New York. 1983. pp 265-176.

110. Otsuka FL, Welch MJ. Evidence for a saturable clearance mechanism for ^{111}In-labeled monoclonal antibodies. *Int. J. Nucl. Med. Biol.* 12:331 1985.

111. Perkins AC, Pimm MV. Differences in tumour and normal tissue concentrations of iodine- and indium-labeled moncoclonal antibody. I. The effect on image contrast in clinical studies. *Eur. J. Nucl. Med.* 11:295 1985.

112. Armitage NC, Perkins AC, Pimm MV, *et al.* The localization of an anti-tumour monoclonal antibody (791T/36) in gastrointestinal tumours. *Br. J. Surg.* 71:407 1984.

113. Teh JG, Stacker SA, Thompson CH, *et al.* The diagnosis of human tumours with monclonal antibodies. *Cancer Surveys* 4:149 1985.

114. Leyden MJ, Thompson CH, Lichtenstein M, *et al.* Visualization of metastases from colon carcinoma using an iodine 131-radiolabeled monoclonal antibody. *Cancer* 57:1135 1986.

115. Chatal J-F, Saccavini J-C, Famoleau P, *et al.* Immunoscintigraphy of colon carcinomas. *J. Nucl. Med.* 25:307 1984.

116. Moldofsky PJ, Sears HF, Mulhern CB, *et al.* Detection of metastatic tumor in normal-sized retroperitoneal lymph nodes by monoclonal-antibody imaging. *N. Engl. J. Med.* 311:106 1984.

117. Moldofsky P, Powe J, Mulhern CB, *et al.* Metastatic colon carcinoma detected with radiolabeled F(ab')$_2$ monoclonal antibody fragments. *Radiology* 149:349 1983.

118. Sears HF, Mattis J, Herlyn D, *et al.* Phase I clinical trial of monoclonal antibody treatment of gastrointestinal tumours. *Lancet* 1:762 1982.

119. Hnatowich DJ, Griffin TW, Kosiuczyk C, *et al.* Pharmacokinetics of an indium-111 labeled monoclonal antibody in cancer patients. *J. Nucl. Med.* 26:849 1985.

120. Smedley HM, Finan P, Lennox ES, *et al.* Localization of metastatic carcinoma by a radiolabelled monoclonal antibody. *Br. J. Cancer* 47:253 1983.

121. Berch C, Mach J-P, Lumbroso J-D, *et al.* Tomoscintigraphy for detecting gastrointestinal and medullary thryroid cancers: first clinical results using radiolabelled monoclonal antibodies against carcinoembryonic antigen. *Br. Med. J.* 285:1447 1982.

122. Epenetos AA, Snook D, Durbin H, *et al.* Limitations of radiolabeled monoclonal antibodies for localization of human neoplasms. *Cancer Res.* 46:3183 1986.

123. Goldenberg DM, Kim EE, Bennett SJ, *et al.* Carcinoembryonic antigen radioimmunodetection in the evaluation of colorectal cancer and in the detection of occult neoplasms. *Gastroenterology* 84:524 1983.

124. Deland FH, Kim EE, Primus FJ, *et al. In vivo* radioimmunodetection of occult recurrent colonic carcinoma. *Am. J. Roentgenol.* 138:145 1982.

125. Fairweather DS, Bradwell AR, Dykes PW, *et al.* Improved tumor localization using indium-111 labeled antibodies. *Br. Med. J.* 287:167 1983.

126. Begent RH, Green AJ, Bagshawe KD, *et al.* Liposomally entrapped second antibody improves tumour imaging with radiolabelled (first) antitumor antibody. *Lancet* 2:739 1982.

127. Jackson PC, Pitcher EM, Davies JO, *et al.* Radionuclide imaging of ovarian tumours with a radiolabelled (^{123}I) monoclonal antibody (NDOG$_2$). *Eur. J. Nucl. Med.* 11:22 1985.

128. Epenetos AA, Hooker G, Durbin H, *et al.* Indium-111 labeled monoclonal antibody to placental alkaline phosphatase in the detection of neoplasms of testis, ovary and cervix. *Lancet* 2:350 1985.

129. Symonds EM, Perkins AC, Pimm MV, *et al.* Clinical implications for immunoscintigraphy in patients with ovarian malignancy: a preliminary study using monoclonal antibody 791T/36. *Br. J. Obstet. Gynaecol.* 92:270 1985.

130. Williams MR, Perkins AC, Campbell FC, *et al.* The use of monoclonal antibody 791T/36 in the immunoscintigraphy of primary and metastatic carcinoma of the breast. *Clin. Oncol.* 10:375 1984.

131. Patiesky N, Philipp K, Skodler WD, *et al.* Radioimmunodetection in pateints with suspected ovarian cancer. *J. Nucl. Med.* 26:1369 1985.

132. Rainsbury RM, Westwood JH, Coombes RC, *et al.* Location of metastatic breast carcinoma by a monoclonal antibody chelate labelled with indium-111. *Lancet* 1:934 1983.

133. Buraggi GL, Callegaro L, Turrin A, *et al.* Immunoscintigraphy with ^{123}I, $^{99}Tc^{m}$ and ^{111}In-labelled $F(ab')_2$ fragments of monoclonal antibodies to a human high molecular weight-melanoma associated antigen. *J. Nucl. Med. Allied Sci.* 28:283 1984.

134. Buraggi GL, Callegaro L, Mariani G, *et al.* Imaging with ^{131}I-labeled monoclonal antibodies to a high molecular-weight melanoma-associated antigen in patients with melanoma: efficacy of whole immunoglobulin and its $F(ab')_2$ fragments. *Cancer Res.* 45:3378 1985.

135. Halpern SE, Hagan P, Witztum KF, *et al.* Radioimmunoscitigraphy using ^{111}In-labeled monoclonal antibodies and the case for immunotherapeutics. *J. Med. Tech.* 2:301 1985.

136. Murray JL, Rosenblum MG, Sobol RE, *et al.* Radioimmunoimaging in malignant melanoma with ^{111}In-labeled monoclonal antibody 96.5. *Cancer Res.* 45:2376 1985.

137. Rosenblum MG, Murray JL, Haynie TP, *et al.* Pharmacokinetics of ^{111}In-labeled anti-p97 monoclonal antibody in patients with metastatic malignant melanoma. *Cancer Res.* 45:2382 1985.

138. Larson SM, Carrasquillo JA, Krohn KA, *et al.* Diagnostic imaging of malignant melanoma with radiolabeled antitumor antibodies. *J. Am. Med. Assoc.* 249:811 1983.

139. Larson SM. Radiolabeled monoclonal anti-tumor antibodies in diagnosis and therapy. *J. Nucl. Med.* 26:538 1985.

140. Larson SM, Carrasquillo JA, McGriffin RW, *et al.* Use of I-131 labeled, murine Fab against a high molecular weight antigen of human melanoma: preliminary experience. *Radiology* 155:487 1985.

141. Javadpour N, Kim EE, Deland FH, *et al.* The role of radioimmunodetection in the management of testicular cancer. *J. Am. Med. Assoc.* 246:45 1981.

142. Armitage NC, Perkins AC, Pimm MV, *et al.* Imaging of bone tumors using a monoclonal antibody raised against human osteosarcoma. *Cancer* 58:37 1986.

143. Sikora K. The characterization of gliomas using human monoclonal antibodies: mini-review on cancer research. *Exp. Cell. Biol.* 52:189 1984.

144. Bullard DE, Bigner DD. Applications of monoclonal antibodies in the diagnosis and treatment of primary brain tumours. *J. Neurosurg.* 63:2 1985.

145. Thiers RE. Contamination in trace metal analysis and its control, in 'Methods of Biochemical Analysis' Vol. V. D Glick, ed. Wiley. New York. 1957. pp 273-335.

146. Vallee BL, Gibson JG. An improved dithizone method for the determination of small quantities of zinc in blood and tissue samples. *J. Biol. Chem.* 176:435 1948.

147. Willard JM, Davis JJ, Wood HG. Phosphoenolpyruvate carboxytransphosphorylase. IV. Requirement for metal cations. *Biochem.* 8:3137 1969.

148. Leung CS-H. The covalent attachment of 'bifunctional' chelates to biomolecules and their uses as physical probes in biological systems. Ph.D. Thesis. University of Calif, Davis. 1977. Order no. 78-9238. University Microfilms, Int. Ann Arbor, Michigan.

149. DeReimer LH, Meares CF, Goodwin DA, *et al.* Bl.EDTA: tumour localization by a bleomycin analogue containing a metal-chelating group. *J. Med. Chem.* 22:1019 1979.

150. Koltun WL. Physicochemical properties of p-carboxyphenylazoinsulins. *J. Am. Chem. Soc.* 19:5681 1957.

151. Currie LA. Quantitative determination - application to radiochemistry. *Anal. Chem.* 40:586 1968.

152. Turner CJ, Sykes TR, Gaudreault RC, *et al.* Determination of metal ion impurities in radiogallium preparations and their effects on the radiolabelling of chelated proteins, in 'Current Applications in Radiopharmacology'. Billinghurst MW, ed. Pergamon Press, Toronto. 1986. pp 309-315.

153. Nelson F, Rush RM, Kraus KA. Anion-exchange studies. XXVII. Adsorbability of a number of elements in HCl-HF solutions. *J. Am. Chem. Soc.* 82:339 1960.

154. Maziere B, Loc'h C, Comar D. *In vitro* preparation of ^{67}Ga-labelled transferrin. *J. Radioanal. Chem.* 76:295 1983.

155. Penefsky HS. A centrifuged-column procedure for the measurement of ligand binding by beef heart F_1, in 'Methods in Enzymology'. Vol 56, part G. Fleischer S, Parker L, ed. Academic Press, New York. 1979. pp 527-530.

156. Rockwell SC, Kallman RF, Fajaido LF. Characteristics of a serially transplanted mouse mammary tumor and its tissue culture adapted derivative. *J. Natl. Cancer Inst.* 49:735 1972.

157. Olivotto M, Bomford R. *In vitro* inhibiton of tumour cell growth and DNA synthesis by peritoneal and lung macrophages from mice injected with *Corynebacterium parvum*. *Int. J. Cancer* 13:478 1974.

158. Gorer PA. Studies in antibody response of mice to tumour innoculation. *Br. J. Cancer* 4:372 1950.

159. MacLean GD, Seehafer J, Shaw ARE, *et al.* Antigenic heterogeneity of human colorectal cancer cell lines analyzed by a panel of monoclonal antibodies. I. Heterogeneous expression of Ia-like and HLA-like antigenic determinants. *J. Natl. Cancer Inst.* 69:357 1982.

160. Leibovitz A, Stinson JC, McCombs WB, *et al.*. Classification of human colorectal adenocarcinoma cell lines. *Cancer Res.* 36:4562 1976.

161. Hauschka TA. Cell population studies on mouse ascites tumours. *Trans. N. Y. Acad. Sci.* 16:64 1953.

162. Margerum DW, Cayley GR, Weatherburn DC, *et al.* in 'Coordination Chemistry. Vol 2.', Martell AE, ed. Am. Chem. Soc. Washington, D.C. 1978. pp 1-220.

163. Kraus KA, Moore GEJ. Anion Exchange Studies. VI. The divalent transition elements manganese to zinc in hydrochloric acid. *J. Am. Chem. Soc.* 75:1460 1953.

164. Kraus KA, Nelson F. Anion exchange studies of metal complexes, in 'The Structure of Electrolytic Solutions', Hamer WJ, ed. John Wiley & Sons. New York. 1959. pp 340-364.

165. Steinkruger FJ, Bentley GE, O'Brien HA. Production and recovery of large quantities of radionuclides for nuclear medicine generator systems, in 'Radionuclide Generators', Knapp FF, Butler TA, eds. Am. Chem. Soc. Washington, D.C. 1984. pp 179-198.

166. Lambrecht RM. Radionuclide generators. *Radiochim. Acta* 34:9 1983.

167. Colombetti LG. Radionuclide generators, in 'Radiotracers for Medical Applications. Vol II', Rayudu GVS, ed. CRC Press, Inc. Boca Raton, Fla. 1983. pp 133-168.

168. Yano Y. Preparation and control of ^{68}Ga radiopharmaceuticals, in 'Radiopharmaceuticals from Generator Produced Radionuclides', IAEA, Vienna. 1971. pp 117-125.

169. Kronvall G, Grey HM, Williams RC, Jr. Protein A reactivity with mouse immunoglobulins: structural relationship between some mouse and human immunoglobulins. *J. Immunol.* 105:1116 1970.

170. Lindmark R, Thorén-Tolling K, Sjöquist J. Binding of immunoglobulins to protein A and immunoglobulin levels in mammalian sera. *J. Immunol. Meth.* 62:1 1983.

171. Ey PL, Prowse SJ, Jenkin CJ. Isolation of pure IgG_1, IgG_{2a}, and IgG_{2b} immunoglobulins from mouse serum using protein A sepharose. *Immunochemistry* 15:429 1978.

172. van Oss CJ. Isolation and characterization of immunoglobulins. *Separation and Purification Methods* 11:131 1982-83.

173. Stephenson JR, Lee JM, Willon-Smith DD. Production and purification of murine monoclonal antibodies: aberrant elution from protein-A sepharose 4B. *Anal. Biochem.* 142:189 1984.

174. Gel filtration: theory and practice. Pharmacia Fine Chemicals. Uppsala, Sweden. 1980. pp 23-29.

175. BioRad price list L. BioRad Laboratories (Canada), Ltd. Mississauga, Ont. 1986. pp 32-39.

176. Saul A, Don M. A rapid method of concentrating proteins in small volumes with high recovery usng sephadex G-25. *Anal. Biochem.* 138:451 1984.

177. Goodwin DA, Meares CF, Diamanti CI, *et al.* Bifunctional chelates for radiopharmaceutical labeling. *Nuklearmedizin* 14:365 1975.

178. Metzger H, Wofsy L, Singer SJ. The participation of A and B polypeptide chains in the active site of antibody molecules. *Proc. Natl. Acad. Sci. (USA)* 51:612 1964.

179. Burton PM, Waley SG. The reaction of triose phosphate isomerase and aldolase with limited amounts of diazonium salts. *Biochem. J.* 104:3P 1967.

180. Kagan HM, Vallee BL. Environmental sensitivity of azo chromophores in arsanilazo carboxypeptidase. *Biochem.* 8:4223 1969.

181. Tabachnick M, Sobotka H. Azoproteins. II. A spectrophotometric study of the coupling of diazotized arsanilic acid with proteins. *J. Biol. Chem.* 235:1051 1960.

182. Higgins HG, Harrington KJ. Reaction of amino acids and proteins with diazonium compounds. II. Spectra of protein derivatives. *Arch. Biochem. Biophys.* 85:409 1959.

183. Yeh SM. I. New bifunctional chelates as biophysical probes. II. Diffusion enhanced lanthanide energy transfer studies of transferrin. Ph.D. Thesis. University of Calif, Davis. 1979. Order no. Z0553 D63. University Microfilms, Int. Ann Arbor, Michigan.

184. Greenwood FC, Hunter WM, Glover JS. The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. *Biochem. J.* 89:114 1963.

185. Bolton AE. Radioiodination techniques. Amersham Radiochemical Centre. Puntarium Ltd. Middlesex. 1977.

186. Boniface GR, Suresh MR, Willans DJ, *et al.*. Renal tubular receptor imaging with iodine-131-labeled peanut lectin: pharmacokinetics and renal clearance mechanism in animals. *J. Nucl. Med.* 27:688 1986.

APPENDIX 1

Percent of injected dose per gram of tissue for Na^{123}I and ^{111}In-citrate in normal Balb/c mice.

Tissue	6 Hours		24 Hours		48 Hours		72 Hours	
	^{111}In	^{123}I	^{111}In	^{123}I	^{111}In	^{123}I	^{111}In	^{123}I
Blood	3.70 ±0.04	0.25 ±0.06	1.27 ±0.13	0.04 ±0.005	0.59 ±0.10	0.02 ±0.002	0.33 ±0.06	0.04 ±0.003
Lung	2.94 ±0.22	0.23 ±0.12	2.61 ±0.29	0.04 ±0.01	1.51 ±0.27	0.02 ±0.001	1.49 ±0.56	0.03 ±0.01
Sal. Gl.	1.93 ±0.08	5.36 ±0.88	2.10 ±0.07	0.42 ±0.10	2.03 ±0.14	0.15 ±0.06	2.19 ±0.63	0.14 ±0.05
Liver	3.30 ±0.12	0.12 ±0.01	4.88 ±0.37	0.05 ±0.01	5.08 ±0.28	0.04 ±0.005	4.49 ±0.66	0.06 ±0.01
Spleen	2.11 ±0.20	0.12 ±0.02	2.77 ±0.22	0.02 ±0.004	2.81 ±0.22	0.01 ±0.001	2.93 ±1.03	0.01 ±0.001
Stomach	0.74 ±0.12	2.72 ±0.89	1.04 ±0.07	0.22 ±0.08	0.86 ±0.15	0.08 ±0.04	0.74 ±0.25	0.07 ±0.01
GIT	1.62 ±0.13	0.28 ±0.05	2.16 ±0.12	0.05 ±0.004	1.79 ±0.16	0.03 ±0.008	1.20 ±0.37	0.05 ±0.01
Kidney	16.34 ±1.56	0.22 ±0.05	17.29 ±1.46	0.08 ±0.02	12.98 ±2.58	0.14 ±0.02	8.87 ±1.00	0.26 ±0.05
Muscle	0.71 ±0.03	0.04 ±0.007	0.60 ±0.02	0.01 ±0.002	0.53 ±0.04	0.01 ±0.002	0.56 ±0.14	0.01 ±0.000
Bone	3.08 ±0.35	0.10 ±0.02	3.47 ±0.36	0.01 ±0.002	3.38 ±0.37	0.01 ±0.001	2.79 ±0.10	0.01 ±0.001

Note: all values are significantly different, $P < 0.01$; $n = 4$.

Percent of injected dose per organ for Na^{125}I and ^{111}In-citrate in normal Balb/c mice.

Organ	6 Hours		24 Hours		48 Hours		72Hours	
	^{111}In	^{125}I	^{111}In	^{125}I	^{111}In	^{125}I	^{111}In	^{125}I
Blood	6.69 ±0.40	0.46 ±0.13	2.22 ±0.17	0.07 ±0.007	1.04 ±0.18	0.04 ±0.008	0.59 ±0.05	0.07 ±0.003
Lung	0.56 ±0.09	0.04 ±0.02	0.43 ±0.07	0.01 ±0.002	0.30 ±0.02	0.004 ±0.00	0.32 ±0.09	0.009 ±0.004
Sal. Gl.	0.36 ±0.02	1.00 ±0.19	0.41 ±0.01	0.08 ±0.02	0.38 ±0.04	0.03 ±0.02	0.41 ±0.09	0.03 ±0.01
Thyroid	0.25 ±0.01	5.44 ±0.64	0.28 ±0.10	5.23 ±1.76	0.26 ±0.05	4.84 ±0.67	0.49 ±0.30	5.58 ±0.55
Liver	4.35 ±0.23	0.16 ±0.03	6.03 ±0.70	0.06 ±0.01	6.14 ±0.91	0.05 ±0.01	5.77 ±0.52	0.08 ±0.008
Spleen	0.24 ±0.03	0.01 ±0.004	0.31 ±0.01	0.002 ±0.00	0.36 ±0.14	0.002 ±0.001	0.33 ±0.08	0.002 ±0.00
Stomach	0.22 ±0.03	0.90 ±0.60	0.22 ±0.05	0.51 ±0.03	0.21 ±0.02	0.02 ±0.01	0.19 ±0.06	0.02 ±0.009
GIT	5.06 ±0.49	0.86 ±0.23	6.21 ±0.36	0.13 ±0.01	5.30 ±0.66	0.09 ±0.04	3.37 ±0.87	0.15 ±0.03
Kidney	7.02 ±0.66	0.09 ±0.03	7.1 ±0.63	0.03 ±0.006	5.23 ±0.51	0.06 ±0.01	3.76 ±0.67	0.11 ±0.007
Muscle	0.33 ±0.05	0.03 ±0.01	0.52 ±0.06	0.007 ±0.002	0.36 ±0.03	0.005 ±0.001	0.51 ±0.15	0.005 ±0.00
Bone	0.34 ±0.05	0.01 ±0.001	0.41 ±0.06	0.009 ±0.00	0.35 ±0.06	0.001 ±0.00	0.38 ±0.09	0.001 ±0.00
Carcass	28.38 ±1.22	3.38 ±1.17	23.54 ±2.54	0.73 ±0.15	20.74 ±0.88	0.36 ±0.03	22.32 ±5.37	0.47 ±0.09
% Dose Rem.	50.64 ±2.49	12.16 ±2.76	46.46 ±4.25	7.45 ±3.70	40.26 ±2.65	5.48 ±0.68	35.17 ±1.46	6.36 ±0.74

Note: all values are significantly different, $P < 0.01$; $n = 4$, except for 6 hour stomach.

Percent of injected dose per gram of tissue for ^{111}I-PNB-EDTA-^{111}In in normal balb/c mice.

Tissue	15 Minutes		1 Hour		6 Hours		24 Hours	
	^{111}In	^{125}I	^{111}In	^{125}I	^{111}In	^{125}I	^{111}In	^{125}I
Blood	4.43 ±0.53	3.56 ±0.47	0.52 ±0.16	0.98• ±0.21	0.08 ±0.01	0.21• ±0.07	0.02 ±0.004	0.02 ±0.005
Lung	3.14 ±0.35	3.11 ±0.57	0.48 ±0.11	0.79• ±0.11	0.08 ±0.01	0.22•• ±0.02	0.05 ±0.01	0.04 ±0.01
Sal. Gl.	1.32 ±0.12	3.04•• ±0.38	0.19 ±0.05	1.83•• ±0.08	0.07 ±0.002	0.50•• ±0.12	0.05 ±0.01	0.03 ±0.02
Liver	2.40 ±0.22	8.95•• ±0.67	0.84 ±0.14	2.61•• ±0.37	0.46 ±0.03	0.65•• ±0.04	0.38 ±0.04	0.35 ±0.05
Spleen	1.18 ±0.09	1.70• ±0.29	0.28 ±0.05	0.70•• ±0.13	0.15 ±0.01	0.24• ±0.06	0.14 ±0.02	0.12 ±0.05
Stomach	1.68 ±0.67	8.74•• ±1.38	0.24 ±0.10	7.28•• ±1.45	0.05 ±0.02	1.08•• ±0.03	0.07 ±0.02	0.07 ±0.04
GIT	1.93 ±0293	3.85•• ±0.41	1.94 ±0.40	4.80 ±0	0.76 ±0.26	1.59 ±0.55	0.16 ±0.05	0.08 ±0.03
Kidney	6.90 ±0.99	8.62 ±1.10	1.92 ±0.2	1.91 0.37	1.15 ±0.12	0.74•• ±0.04	0.51 ±0.07	0.33• ±0.06
Muscle	0.91 ±0.08	0.87 ±0.07	0.16 ±0.07	0.29 ±0.09	0.03 ±0.001	0.04•• ±0.001	0.02 ±0.003	0.01• ±0.004
Bone	2.50 ±0.25	1.68• ±0.26	0.55 ±0.08	0.57 ±0.08	0.16 ±0.04	0.20 ±0.04	0.13 ±0.02	0.07•• ±0.01

Note: all values are x ± s.d., n = 4.
• $P < 0.05$; •• $P < 0.01$.

Percent of injected dose per organ for ^{125}I-PNB-EDTA-^{111}In in normal Balb/c mice.

Organ	15 Minutes		1 Hour		6 Hours		24 Hours	
	^{111}In	^{125}I	^{111}In	^{125}I	^{111}In	^{125}I	^{111}In	^{125}I
Blood	5.68 ±0.35	4.56 ±0.31	0.72 ±0.22	1.37* ±0.28	0.11 ±0.01	0.29* ±0.10	0.03 ±0.002	0.03 ±0.009
Lung	0.52 ±0.03	0.52 ±0.04	0.09 ±0.05	0.13 ±0.02	0.01 ±0.004	0.04** ±0.007	0.009 ±0.001	0.008 ±0.002
Sal. Gl.	0.17 ±0.03	0.39** ±0.07	0.03 ±0.004	0.24** ±0.03	0.009 ±0.001	0.07** ±0.02	0.006 ±0.001	0.004 ±0.002
Thyroid	0.07 ±0.01	0.27** ±0.01	0.01 ±0.004	0.55** ±0.08	0.002 ±0.0001	1.15** ±0.12	0.002 ±0.0001	1.74* ±0.97
Liver	2.17 ±0.27	8.09** ±0.94	0.86 ±0.20	2.75** ±0.53	0.44 ±0.02	0.62** ±0.05	0.41 ±0.05	0.38 ±0.03
Spleen	0.12 ±0.02	0.17** ±0.01	0.03 ±0.01	0.08** ±0.01	0.02 ±0.001	0.03* ±0.01	0.02 ±0.002	0.01* ±0.01
Stomach	0.41 ±0.07	2.22** ±0.44	0.07 ±0.02	2.09** ±0.69	0.01 ±0.004	0.28** ±0.10	0.02 ±0.01	0.02 ±0.01
GIT	4.62 ±0.31	9.24** ±0.77	4.69 ±0.88	11.96** ±2.07	1.78 ±0.58	3.74* ±1.23	0.41 ±0.14	0.22 ±0.09
Kidney	1.76 ±0.26	2.20 ±0.18	0.51 ±0.07	0.51 ±0.10	0.28 ±0.01	0.18** ±0.01	0.15 ±0.02	0.10 ±0.03
Muscle	0.55 ±0.03	0.52 ±0.01	0.10 ±0.04	0.19 ±0.05	0.02 ±0.002	0.03** ±0.003	0.01 ±0.001	0.01 ±0.002
Bone	0.25 ±0.06	0.17 ±0.05	0.07 ±0.01	0.07 ±0.01	0.02 ±0.003	0.02 ±0.003	0.01 ±0.001	0.01 ±0.001
Carcass	26.60 ±2.17	22.44* ±1.15	4.30 ±1.16	7.80*3 ±1.64	0.95 ±0.16	1.66 ±0.54	0.59 ±0.12	0.31* ±0.05
% Dose Rem.	39.77 ±3.04	48.15** ±3.61	11.10 ±2.22	27.06** ±5.09	3.60 ±0.65	7.99** ±1.84	1.65 ±0.31	2.80 ±1.11

Note: all values are x ± s.d., n = 4
* $P < 0.05$; ** $P < 0.01$.

Percent of injected dose per gram of tissue for ^{125}I-poly-H-IgG and ^{111}In-B-EDTA-poly-H-IgG in normal Balb/c mice.

Tissue	6 Hours		24 Hours		48 Hours		72 Hours	
	^{111}In	^{125}I	^{111}In	^{125}I	^{111}In	^{125}I	^{111}In	^{125}I
Blood	26.44 ±6.09	32.26 ±6.94	18.83 ±2.19	22.68* ±2.42	16.36 ±1.85	20.07* ±2.12	13.46 ±1.46	16.81* ±1.66
Lung	12.40 ±2.71	15.48 ±4.52	9.35 ±1.96	11.72 ±2.53	9.54 ±1.73	11.80 ±2.55	8.36 ±1.20	10.51 ±1.70
Sal. Gl.	3.72 ±0.38	6.50** ±1.13	4.39 ±1.01	5.39 ±1.14	3.58 ±0.76	4.09 ±0.93	3.59 ±0.48	3.79 ±0.50
Liver	32.34 ±6.65	6.02** ±1.30	26.50 ±3.37	4.02** ±0.39	21.92 ±1.66	3.34** ±0.48	18.26 ±4.80	2.94** ±0.28
Spleen	14.27 ±4.26	7.12* ±1.55	11.37 ±1.23	5.10** ±0.50	10.63 ±1.04	4.45** ±0.44	8.40 ±1.92	3.50** ±0.64
Stomach	1.89 ±0.53	6.64** ±1.70	1.143 ±0.46	2.26* ±0.52	1.16 ±0.26	1.64* ±0.30	1.41 ±0.39	1.83 ±0.51
GIT	3.76 ±0.74	3.82 ±0.74	2.94 ±0.30	2.57 ±0.31	2.26 ±0.13	2.05 ±0.17	2.10 ±0.15	1.81* ±0.10
Kidney	10.16 ±1.99	7.73 ±1.30	9.11 ±0.68	5.24** ±0.44	8.05 ±1.06	4.19** ±0.52	6.99 ±1.21	3.43** ±0.40
Muscle	1.26 ±0.27	1.72 ±0.31	1.45 ±0.09	1.80** ±0.12	1.28 ±0.22	1.58 ±0.24	1.08 ±0.13	1.15 ±0.37
Bone	3.83 ±0.81	3.80 ±0.71	4.43 ±0.50	2.80** ±0.11	4.95 ±0.97	2.02** ±0.35	4.92 ±1.04	1.80** ±0.14

Note: all values are $\bar{x} \pm$ s.d.; $n = 5$.
* $P < 0.05$; ** $P < 0.01$.

Percent of injected dose per organ for ^{125}I-poly-H-IgG and ^{111}In-B-EDTA-poly-H-IgG in normal Balb/c mice.

Organ	6 Hours		24 Hours		48 Hours		72 Hours	
	^{111}In	^{125}I	^{111}In	^{125}I	^{111}In	^{125}I	^{111}In	^{125}I
Blood	30.72 ±6.44	37.50 ±7.28	23.72 ±2.26	28.59* ±2.59	20.74 ±1.84	25.44** ±1.91	17.30 ±2.36	21.59* ±2.58
Lung	2.11 ±0.82	2.65 ±1.17	1.50 ±0.39	1.88 ±0.51	1.76 ±0.44	2.16 ±0.58	1.60 ±0.49	2.04 ±0.71
Sal. Gl.	0.43 ±0.02	0.75** ±0.07	0.50 ±0.12	0.62 ±0.14	0.44 ±0.07	0.50 ±0.08	0.43 ±0.09	0.45 ±0.09
Thyroid	0.16 ±0.06	1.47** ±0.49	0.15 ±0.07	2.32* ±1.31	0.11 ±0.02	2.10** ±1.10	0.14 ±0.07	3.49** ±1.27
Liver	30.67 ±4.80	5.72** ±0.99	26.78 ±3.33	4.06** ±0.33	24.88 ±4.06	3.75** ±0.38	20.99 ±5.77	3.38** ±0.36
Spleen	1.64 ±0.32	0.83** ±0.13	1.40 ±0.24	0.63** ±0.10	1.39 ±0.25	0.58** ±0.08	1.15 ±0.32	0.48** ±0.10
Stomach	0.39 ±0.08	1.45** ±0.57	0.40 ±0.06	0.63** ±0.04	0.32 ±0.07	0.46 ±0.10	0.29 ±0.06	0.38 ±0.08
GIT	8.71 ±1.31	8.84 ±1.23	6.67 ±0.74	5.84 ±0.74	5.81 ±0.51	5.26 ±0.32	5.47 ±0.76	4.71 ±0.61
Kidney	2.52 ±0.23	1.93** ±0.19	2.37 ±0.11	1.36** ±0.09	2.05 ±0.25	1.07** ±0.12	1.77 ±0.30	0.87** ±0.12
Muscle	0.72 ±0.29	0.99 ±0.36	0.91 ±0.09	1.13* ±0.11	0.74 ±0.13	0.91 ±0.14	0.65 ±0.13	0.70 ±0.27
Bone	0.42 ±0.07	0.42 ±0.09	0.44 ±0.05	0.28** ±0.04	0.49 ±0.13	0.21** ±0.10	0.48 ±0.06	0.18** ±0.03
Carcass	40.31 ±5.45	47.01 ±6.47	45.88 ±3.77	48.53 ±3.33	40.47 ±2.07	42.60 ±2.43	34.57 ±3.39	36.25 ±3.61
% Dose Rem.	102.65 ±13.14	89.81 ±11.46	97.68 ±6.80	79.23** ±4.89	87.43 ±7.07	70.56** ±2.72	70.57 ±7.43	61.96 ±6.84

Note: all values are x ± s.d., n = 5
* $P < 0.05$; ** $P < 0.01$.

Percent of injected dose per gram of tissue for ^{131}I-poly-H-IgG, ^{131}I-MAb-155H.7 and ^{111}In-B-EDTA-MAb-155H.7.

Tissue	6 Hours			24 Hours			48 Hours			72 Hours		
	^{111}In	^{131}I	^{125}I	^{111}In	^{131}I	^{125}I	^{111}In	^{131}I	^{125}I	^{111}In	^{131}I	^{125}I
Blood	16.15* ±0.78	25.33 ±1.64	26.42 ±1.26	5.53* ±0.53	11.16 ±1.45	13.58 ±1.47	3.75** ±0.29	7.30 ±1.08	11.88 ±0.90	3.25** ±0.71	5.33 ±1.14	11.30 ±1.89
Lung	8.32* ±1.23	10.75 ±0.98	12.01 ±2.04	3.96 ±0.38	4.68 ±0.52	5.70* ±1.02	3.60 ±0.21	2.87 ±0.64	5.33* ±1.48	3.11 ±0.49	2.53 ±0.93	4.74* ±0.17
Sal. Gl.	2.41** ±1.02	19.11 ±4.78	8.22 ±2.39	2.76* ±0.99	12.14 ±8.10	7.00 ±2.95	3.39 ±0.68	2.60 ±1.03	3.21 ±0.23	3.25 ±0.48	1.62* ±0.32	2.63 ±0.39
Liver	6.10 ±0.47	7.71* ±0.88	5.99 ±0.26	5.81* ±0.59	2.99 ±0.44	2.87 ±0.52	5.76* ±0.44	1.56 ±0.28	2.04 ±0.14	5.70** ±0.54	1.28 ±0.19	2.05 ±0.33
Spleen	5.04* ±0.34	4.00 ±0.57	3.71 ±0.11	5.75* ±0.75	2.18 ±0.73	1.93 ±0.21	5.69** ±0.47	0.47 ±0.03	1.54 ±0.14	4.86** ±0.90	0.50 ±0.10	1.46 ±0.26
Stomach	1.33** ±0.41	11.92 ±3.33	4.94 ±0.99	1.00 ±0.13	5.48* ±2.39	2.92 ±1.12	0.71** ±0.17	1.24 ±0.13	1.04 ±0.11	0.62 ±0.16	0.75 ±0.20	0.90 ±0.29
GIT	2.36** ±0.17	2.97 ±0.44	1.90 ±0.18	2.42* ±0.54	1.32 ±0.45	1.10 ±0.20	1.91* ±0.16	0.66 ±0.09	0.78 ±0.08	1.45** ±0.36	0.45 ±0.09	0.78 ±0.17
Kidney	18.25* ±2.13	4.65 ±1.14	5.85 ±0.55	20.19** ±4.09	5.40 ±0.32	3.24 ±0.54	19.08** ±2.17	5.85 ±0.55	2.88 ±0.45	13.83* ±2.13	2.72 ±0.98	2.40 ±0.50
Muscle	1.06* ±0.14	0.81 ±0.13	0.83 ±0.14	0.79 ±0.09	0.54* ±0.09	0.69 ±0.07	0.81** ±0.04	0.38 ±0.05	0.71 ±0.02	0.76 ±0.12	0.33* ±0.06	0.66 ±0.12
Bone	3.05** ±0.56	1.28 ±0.33	1.75 ±0.17	3.27* ±0.44	1.50 ±0.32	1.04 ±0.15	3.28** ±0.38	0.31 ±0.02	0.90 ±0.08	2.68** ±0.70	0.23 ±0.02	0.83 ±0.13
Tumour	8.58 ±0.60	8.85 ±1.49	8.58 ±0.44	7.75 ±1.10	6.99 ±1.36	6.88 ±1.61	8.08** ±0.17	4.45 ±0.80	6.02 ±0.61	7.75** ±0.59	3.61 ±0.87	5.51 ±0.64

Note: all values are x ± s.d., n = 5

* Value is significantly different from the other two, $P < 0.05$.

** Values are significantly different from each other, $P < 0.05$.

Percent of injected dose per organ for ^{131}I-poly-H-IgG, ^{131}I-MAb-155H.7 and ^{111}In-B-EDTA-MAb-155H.7 in TA3/Ha tumour bearing mice.

Organ	6 Hours			24 Hours			48 Hours			72 Hours		
	^{111}In	^{131}I	^{125}I	^{111}In	^{131}I	^{125}I	^{111}In	^{131}I	^{125}I	^{111}In	^{131}I	^{125}I
Blood	13.70* ±2.68	21.47 ±2.31	22.62 ±3.98	9.63 ±0.78	8.89 ±1.72	10.65 ±1.17	2.98** ±0.59	5.86 ±1.70	9.43 ±1.87	2.77** ±0.36	4.58 ±0.84	9.66 ±0.92
Lung	1.69 ±0.43	2.19 ±0.53	2.45 ±0.70	0.78* ±0.12	0.91 ±0.09	1.11 ±0.18	0.82 ±0.18	0.54* ±0.07	0.96 ±0.09	0.67** ±0.18	0.43 ±0.07	0.96 ±0.08
Sal. Gl.	0.43** ±0.18	3.40 ±0.76	1.46 ±0.39	0.46* ±0.18	1.98 ±1.23	1.16 ±0.44	0.60 ±0.14	0.45 ±0.17	0.57 ±0.02	0.58 ±0.08	0.29* ±0.04	0.47 ±0.06
Thyroid	0.16** ±0.07	2.05 ±0.66	0.85 ±0.32	0.08** ±0.02	2.40 ±0.29	0.97 ±0.10	0.07** ±0.04	3.09 ±0.39	1.40 ±0.11	0.001** ±0.00	3.12 ±0.45	1.60 ±0.16
Liver	7.04 ±0.64	8.86* ±0.73	6.90 ±0.33	7.09* ±0.47	3.64 ±0.28	3.48 ±0.42	8.24* ±0.72	2.26 ±0.55	2.92 ±0.30	8.57** ±0.97	1.74 ±0.29	2.76 ±0.08
Spleen	1.41* ±0.23	1.11 ±0.18	1.03 ±0.10	1.70* ±0.26	0.65 ±0.24	0.57 ±0.05	1.86** ±0.24	0.16 ±0.03	0.50 ±0.05	1.48** ±0.18	0.15 ±0.02	0.44 ±0.06
Stomach	0.33** ±0.07	3.03 ±0.76	1.26 ±0.31	0.31* ±0.06	1.57 ±0.68	0.85 ±0.34	0.29* ±0.03	0.53 ±0.14	0.44 ±0.08	0.25 ±0.03	0.26 ±0.05	0.30 ±0.03
GIT	6.37** ±0.32	8.00 ±0.78	5.42 ±0.34	6.10* ±0.95	3.36 ±0.91	3.33 ±1.10	5.64** ±0.45	1.94 ±0.15	2.31 ±0.27	4.44** ±0.67	1.38 ±0.14	2.40 ±0.27
Kidney	6.83* ±0.99	1.73 ±0.38	2.19 ±0.25	7.77** ±1.83	2.06 ±0.02	1.24 ±0.02	7.06** ±0.50	2.16 ±0.01	1.06 ±0.13	5.30* ±0.88	1.04 ±0.36	0.92 ±0.17
Muscle	0.80* ±0.14	0.61 ±0.09	0.63 ±0.11	0.60 ±0.08	0.41* ±0.06	0.53 ±0.08	0.63** ±0.06	0.30 ±0.04	0.54 ±0.03	0.60 ±0.11	0.26* ±0.04	0.51 ±0.06
Bone	0.33 ±0.07	0.14 ±0.04	0.18 ±0.02	0.38* ±0.06	0.18 ±0.05	0.12 ±0.02	0.33* ±0.06	0.03 ±0.01	0.09 ±0.01	0.27* ±0.04	0.02 ±0.004	0.08 ±0.01
Tumour	2.77 ±1.51	3.12 ±2.38	2.83 ±1.70	5.74 ±1.71	5.35 ±2.27	5.10 ±1.84	4.78* ±0.64	2.69 ±0.73	3.36 ±0.69	3.27 ±1.19	1.31 ±0.88	1.95 ±1.15
Carcass	44.89* ±4.11	37.19 ±5.71	36.76 ±3.36	43.96 ±3.73	32.51* ±7.37	40.63 ±3.68	42.55** ±4.19	19.51 ±2.66	35.19 ±1.39	37.17** ±2.07	14.23 ±1.94	28.76 ±2.70
%Dose Rem.	87.46 ±6.40	93.52 ±8.53	85.03 ±5.86	79.53 ±4.35	68.3 ±16.75	71.38 ±3.24	76.04** ±4.51	39.82 ±5.47	59.41 ±0.92	65.13** ±3.97	29.08 ±3.74	51.43 ±3.32

Note: all values are x ± s.d., n = 5

* Value is significantly different from the other two, $P < 0.05$

** Values are significantly different from each other, $P < 0.05$

APPENDIX 2

STATISTICAL ANALYSIS

All statistical analysis of biodistribution data was performed using the Student's paired t-test. This test determines if the difference between two means is statistically significant. The basic assumption for this test is that the data to be analyzed is not different i.e. mean 1 = mean 2 and if this null hypothesis does not hold true, then the difference between the two means is determined to be significant. For paired data, all sources of experimental error other than treatment must be the same for each pair and the mathematical equation employed is:

$$t = \frac{\bar{X}_d}{S_{\bar{d}}}$$

where

$\bar{X}_d$ = mean sample difference (absolute)

$S_{\bar{d}}$ = standard error of the mean difference

and

$$S_{\bar{d}} = \sqrt{\frac{\Sigma(X_1 - X_2)^2 - \frac{(\Sigma X_1 - \Sigma X_2)^2}{n}}{n(n-1)}}$$

where

n = number of observations

X_1 = mean 1

X_2 = mean 2

The interpretation of the t value obtained by this test is generally determined from standard tables of probability versus t. The degrees of freedom involved and the confidence limits required the [illegible] significance of the difference observed between the two means. The degree[illegible]ers to the number of independent random selections that

may be made from a sample and for the paired t-test is equivalent to n - 1, where n is the number of observations. The confidence limits correspond to values that are considered to give reasonable assurance that the true value being estimated lies within these limits. The confidence limit for the present studies was chosen to be 95% or greater corresponding to probability levels of 0.05 or less. The actual calculations were performed with the aid of a computer program (TEE) generated by C. Ediss on the University of Alberta Ahmdahl computer to expedite analysis.

TRIPLE LABEL CROSSOVER CORRECTION PROGRAM

This program was generated by C. Ediss for use with a Tracor TN 1117 computer interfaced to a Tracor 2200 multi-sample NaI(Tl) detector (Tracor-Northern). It was initially developed for ^{131}I, ^{125}I and ^{67}Ga but was easily adapted to use with ^{131}I, ^{125}I and ^{111}In for the work presented here.

The program requires that components of each radionuclide can be isolated from interference by each of the other radionuclides involved. The program itself has two parts, an initial calibration step followed by analysis of sample radioactivity. In the calibration step, three windows are selected manually and each is set for the most favourable detection of one of the radionuclides. Three standards consisting of ^{131}I, ^{125}I or ^{111}In are prepared such that their count rate is in the same approximate range as the samples to be analyzed. Each standard is then counted in all three windows along with a background sample to give a 3 x 3 matrix of counting efficiencies. This matrix is stored in the computer memory and as the samples are analyzed, the inverse of this matrix is used to correct the count rates of the samples for spillover in the three windows. Further matrix manipulation provides an estimate of the error in the corrected sample count rates due to background and the statistical contribution of the spillover correction technique.

As an added feature, the program can correct each sample for physical decay of the radioisotopes and automatically subtracts background making further mathematical manipulation of the data infinitely easier.