NOTICE

The quality of this microform 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.

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

AVIS

La qualité de cette microforme 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.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

THE UNIVERSITY OF ALBERTA

Radioscintigraphic Detection of T-Antigen Expressing Tumors with Radiochelated Peanut Lectin

by

Thomas K. Woo

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 (Radiopharmacy)

Faculty of Pharmacy and Pharmaceutical Sciences

EDMONTON, ALBERTA

Spring 1989

ISBN 0-315-52840-0

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR Thomas K. Woo

TITLE OF THESIS Radioscintigraphic Detection of T-Antigen Expressing Tumors with Radiochelated Peanut Lectin

DEGREE FOR WHICH THESIS WAS PRESENTED Master of Science

YEAR THIS DEGREE GRANTED Spring 1989

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) .....Thomas Woo.....

PERMANENT ADDRESS:

.....1023 101 Street.....

.....Edmonton Alberta.....

.....Canada T6J 6H3.....

DATED December 14, 1988

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 Radioscintigraphic Detection of T-Antigen Expressing Tumors with Radiochelated Peanut Lectin submitted by Thomas K. Woo in partial fulfilment of the requirements for the degree of Master of Science in Pharmaceutical Sciences (Radiopharmacy).

.....

Supervisor

.....

.....

.....

Date December 14/1988

To Eliza

Abstract

The Thomsen-Friedenreich (T) antigen in its exposed form is found on the surface of many human adenocarcinoma but is masked by sialic acid on normal healthy tissues. Peanut lectin agglutinin (PNA) binds avidly to the immunodeterminant structure (β-D-galactosyl-α-(1→3) N-acetyl-D-galactosamine) of the T-antigen and has been investigated as a potential radiopharmaceutical in the detection of T-antigen expressing solid tumors and metastases. High tumor uptake and clear delineation of the TA_3/Ha solid adenocarcinoma in a mouse animal model are observed with radioiodinated PNA. However, rapid *in vivo* deiodination of this radiotracer occurs.

Chelation of radioactive metal ions *via* bifunctional chelating agents is a feasible alternative to radioiodination. Indium-111 is attached to PNA by using the bifunctional chelating agent DTPA cyclic dianhydride. The conjugation reaction between PNA and DTPA anhydride is affected by the pH of the buffer, the PNA concentration and the molar ratio between PNA and DTPA dianhydride. With a starting PNA concentration of 20 mg/ml and a 10 to 1 DTPA/PNA molar ratio at pH 8.5, a kit preparation of DTPA-PNA can be obtained with high In-111 chelating capacity. The radiolabelling with In-111 can be achieved by the simple addition of In-111 citrate to the DTPA-PNA kit. The In-111 DTPA-PNA prepared is found to retain its T-antigen binding ability by binding to neuraminidase-treated red blood cells, various T-antigen expressing tumor cell lines, and to an immobilized synthetic T-antigen disaccharide (T-Synsorb). The In-111 DTPA-PNA is stable for at least 3 days at room temperature and is resistant to transchelation to an excess of free DTPA or EDTA. Gradual loss of the In-111 label was noted upon incubation with serum.

Biodistribution studies with In-111 DTPA-PNA in CAF_1/J mice bearing the TA_3/Ha tumor revealed high tumor uptake of the radiotracer but uptake in various non-target organs, especially liver and kidneys, is also very high. Maximum tumor uptake of 8.96% injected dose/gram of tumor is achieved at 3 hours post injection. The tumor uptake of In-111 DTPA-PNA is higher than that observed with I-125 PNA but the slow whole body clearance

of In-111 DTPA-PNA decreased the tumor to non-target ratio of the radiotracer. The different biodistribution patterns of In-111 citrate and In-111 DTPA-PNA in the same animal model confirmed the specificity of tumor uptake of the radiolabelled lectin.

Whole body radioscintigraphic imaging with In-111 DTPA-PNA in the TA$_3$/Ha murine tumor model revealed good delineation of the tumor as early as 6 hours post-injection without the need for blood background subtraction techniques. No uptake in the thyroid was observed.

The renal elimination of In-111 DTPA-PNA in NZW rabbits ag[illegible]wed higher retention of the radiolabel in the kidneys as compared to I-131 PNA. Following I.V. injection, intact In-111 DTPA-PNA was detected in the urine of Sprague Dawley rats together with some smaller molecular weight forms which do not possess affinity for the T-antigen. This may reflect the metabolic breakdown of In-111 DTPA-PNA in the kidneys.

Further studies with newer chelates which are metabolizable in the liver may decrease the non-target uptake of In-111 labelled PNA and enhance the contrast between the tumor and the non-target tissues.

Acknowledgements

I would like to take this opportunity to express my sincere gratitude to my supervisor, Dr. Alec Shysh, for his support and guidance throughout the course of the research and in the preparation of this manuscript, without whose help and patience this work would not have been possible.

I wish to thank Drs. A.A. Noujaim and T.R. Sykes for their valuable ideas and help with the radiochelation project; Peter Eu for his helpful guidance in the laboratory; Kent Leung for his help with the animal work; Chris Ediss and Steve McQuarrie for their technical assistance; and Dr. M.R. Suresh for his expert advice and consultations.

The helpful discussions and friendship of the students, staff and associates of the Division of Bionucleonics and Radiopharmacy, especially Dr. Yip W. Lee, Brad Hunter, Lihua Xu, Takashi Iwashina, Pei Qi, Alicja Borowski and Barry Williams, are greatly appreciated.

The financial support of the Alberta Heritage Foundation for Medical Research in the form of a studentship is gratefully acknowledged.

Table of Contents

List of Tables

List of Figures

1. INTRODUCTION

Many different radiolabelled compounds have been developed and investigated for use in radioimaging of malignant neoplasia. The design of these radiopharmaceuticals was aimed at achieving affinity to tumor cells based on certain unique characteristics of tumor cells as opposed to the normal cells in the host. One of these features is the presence of tumor cell surface antigens which results from the alteration of certain cell surface properties, such as the expression of various carbohydrate chains, during the neoplastic transformation.

The Thomsen-Friedenreich (T) antigen is a common surface antigen found in a non-cryptic form on many human adenocarcinomas but normally not on other healthy tissues. The detection of this T-antigen is currently of great interest for the early diagnosis of certain cancers. Peanut Agglutinin (PNA) has been shown to have an avid and specific binding affinity for the immunodeterminant structure (β-D-galactosyl-α-(1→3) N-acetyl-D-galactosamine) of the T antigen. In previous studies, clear delineation of the T-antigen expressing TA_3/Ha mammary adenocarcinoma in strain A mice had been obtained with radioiodinated PNA.

A significant problem, however, with most radioiodinated proteins is that they undergo rapid *in vivo* deiodination resulting in the release of free radioiodide into the circulation. The redistribution of the free radioiodide to other non-malignant tissues can severely interfere with the diagnostic quality of the scan. Although radioiodinated PNA localized readily in experimental tumor models, rapid *in vivo* deiodination was observed.

Another recent approach to the radiolabelling of proteins involves the radiochelation of radioactive metal ions *via* the use of bifunctional chelating agents. Studies with a variety of proteins, especially radiochelated antibodies showed that the biological activity can be retained with proper radiolabelling procedures.

The aim of this project was to prepare In-111 radiolabelled PNA with the expectation of circumventing the dehalogenation problems of radioiodinated PNA. The efficiency of the radiochelation reaction is influenced by the amino-acid composition of each particular

protein, thus it was necessary to establish the optimal conditions for radiochelation of indium-111 to PNA *via* the bifunctional chelating agent DTPA dianhydride. Finally, a comparison was made between In-111 DTPA-PNA , I-125 PNA and In-111 citrate with respect to their *in vitro* binding characteristics and their *in vivo* biodistribution in a TA$_3$/Ha animal tumor model.

2. SURVEY OF THE LITERATURE

2.1 Tumor Imaging

Tumor localization by the administration of radiolabelled tracers provides a non-invasive method for the early detection of tumors and also provides a simple means for evaluation and follow up on the efficacy of the treatment modalities[1].

The tumor must show a preferential accumulation of the radiotracer over the normal tissues to allow for a clinically useful scan. It has been suggested that a tumor to background ratio of 2:1 is required for the detection of a tumor with a 2 inch diameter[2]. Smaller tumors will need a higher tumor to background ratio in order to be visualized[2].

Malignant and normal tissue differ in many characteristics , and the tumor localizing radiotracers utilize one or more of these characteristics as their mechanism for localization. Due to the complexity between the nature of normal and malignant tissue, it is quite unlikely that any single radiopharmaceutical will be developed which will localize in all cancerous lesions[2]. Some examples of commonly employed tumor localizing radiopharmaceuticals are presented in the following section.

2.1.1 Indirect Localizing Radiopharmaceuticals

Tumor localizing radiopharmaceuticals which utilize the indirect method of tumor uptake depend on cancer induced alterations in normal tissue function and structure thus allowing a diagnostic scan. These radiopharmaceuticals are non-specific in their action since they also localize in other non-malignant tissues. They do not have a direct affinity for the tumor cells as such, and their uptake is due to the physiological changes induced by the presence of tumor. Some examples of this type of radiopharmaceutical include: brain imaging with Tc-99m sodium pertechnetate in areas with increased permeability in the blood brain barrier[3]; Tc-99m phosphates which localize in bone with increased osteoblastic activity[4]; and radiolabelled colloids which show decreased localization in areas of liver with parenchymal

destruction[5].

2.1.2 Direct Localizing Radiopharmaceuticals

Direct localizing radiopharmaceuticals are taken up preferentially *in vivo* by the tumor cell itself. They are either a metabolite which is taken up by the tumor cells, or they have an affinity for certain unique characteristics of the tumor cells which are different from non-malignant tissues[6,7].

Many tumor cells have a higher rate of metabolism due to the increased rate of cell proliferation, thus they have a higher requirement for metabolites[7]. Radiolabelled metabolites can be tailored to a particular tumor. I-131 iodocholesterol accumulates in cortical secreting adenomas[1], I-131 sodium iodide is taken up to a large extent by hyperactive thyroid tissues[7], and Se-75 selenomethionine localizes in hepatomas and lymphomas with increased protein synthesis[1,8]. Other radiolabelled amino acid and sugar analoges have been investigated as tumor scanning agents, but they fail to interact with a broad spectrum of tumor types[1].

Antibiotics with antitumor activity such as bleomycin, adriamycin, tetracycline and streptozotocin are thought to exert their effects on tumor cells. Therefore, they have been radiolabelled with various radioisotopes for use in tumor detection and enhancement of therapeutic effects[9]. Much of the research has been done on bleomycin, which has been labelled with Tc-99m, Ga-67, Co-57, In-111, I-123 and Au-198 without loss in activity[9]. However, the radiolabel is not stable *in vivo*, and bleomycin is found to localize in other non-malignant processes such as infection. Clinical utilization of these compounds is limited due to a low tumor to background ratio[7,9].

Despite its disadvantages, gallium-67 citrate is commonly used as a tumor localization agent at the present time. The use of gallium-67 citrate in clinical studies has recently been reviewed by Bekerman *et al* [10]. Gallium-67 citrate is a broad spectrum tumor localizing agent with good uptake in certain tumor types, such as bronchogenic carcinoma, lymphoma and Hodgkin's disease, hepatoma and melanoma, but this agent is not very useful for detection of

breast, gastrointestinal and genitourinary tumors [10,11]. The biodistribution of gallium-67 citrate is somewhat complex, with an increased uptake in various inflammatory diseases as well as localization in a wide variety of benign tissues. This non-specificity has limited the diagnostic value of gallium-67 citrate in tumor imaging[10].

Recently, radiolabelled antibodies, based on their specific affinity for tumor associated markers have been extensively investigated. The specificity of the antibody-antigen interaction provides a potential tool for the differentiation between malignant and normal tissues based on the binding to antigens which appear after the malignant transformation[12,13]. Some of the progress that has been made in the field of radiolabelled antibodies for tumor localization is reviewed in the following section.

2.1.3 Radiolabelled Antibodies

The first reported use of radiolabelled antibodies for tumor localization was by Pressman and Korngold in 1953[14,15]. Since then, many researchers have studied the application of a variety of radiolabelled antibodies for tumor detection and therapy[16,17].

In the early days, polyclonal antibodies, which were produced by immmunizing the host animal with whole tumor cells or cell fragments, were used for the studies. Due to the heterogeneous nature of the antibodies, a high degree of reactivity with normal tissues was seen when these antibodies were used *in vivo*[18]. This necessitated the use of background subtraction techniques to visualize the localization of the tracer[19,20]. The use of certain purification techniques such as tissue absorption and affinity chromatography improved the quality of the antibodies but failed to significantly increase the quality of the scans[21].

In 1975, Kohler and Milstein[22] developed the hybridoma technology that allows the production of highly specific monoclonal antibodies. This technique revolutionized the field of radioimmunodetection by providing highly specific antibodies which can be raised against specific tumor antigens and can be produced in large amounts (up to several grams).

The monoclonal antibodies have been labelled with a wide variety of radioisotopes for radioimmunodetection or therapy (see table 1 and 2). Radiolabelled monoclonal antibodies have also been studied in a wide range of human malignancies including melonoma, ovarian cancer, testicular cancer, colorectal carcinoma, germ cell tumors and tumors of the nervous system[23,24]. Background subtraction techniques are not usually necessary with many current monoclonal antibody preparations which yield tumor to tissue ratios of up to 7:1 in patients and of up to 20:1 in mice[23].

It has generally been recognized that there are a number of factors which will influence the localization of the radiolabelled monoclonal antibody in the host tumor and these include:[23-25]

1. The affinity of the antibody to the antigen.
2. The concentration of the antigen on the tumor.
3. The isolation of tumor specific antigens.
4. The mass amount of radiolabelled monoclonal antibody administrated.
5. The route of administration of the radiolabelled monoclonal antibody.
6. The use of radiolabelling and quality control procedures that allow the retention of immunological reactivity.
7. The use of either IgG, $F(ab')_2$ or Fab fragments to increase the rate of blood clearance.
8. The use of different monoclonal antibodies in combination.

Table 3 summarizes some of the radiolabelled antibodies that have been used in the study of human cancers.

2.2 Tumor Associated Markers

The binding of radiolabelled tracer compounds to tumor-associated markers is clinically utilized for the early identification of neoplasias and monitoring of treatment. Tumor-associated markers arise from the neoplastic transformation of normal tissue and are

Table 1. Characteristics of Some Radionuclides Used for Radioimmunoimaging

Radioisotope	Half-Life	Decay mode	Major γ energy
Tc-99m	6 hr	I.T.[1]	141 KeV(89%)
I-123	13.2 hr	E.C.[2]	159 KeV(82.8%)
I-131	8.0 d	β^-	31 KeV(16.0%)
			364 KeV(81.2%)
Ga-67	3.3 d	E.C.[2]	93 KeV(38.3%)
			185 KeV(20.9%)
			300 KeV(16.8%)
Ga-68	68.0 min	β^+	511 KeV(178.2%)
		E.C.[2]	
In-111	2.8 d	E.C.[2]	17? KeV(90.0%)
			247 KeV(94.2%)
In-113m	99.5 min	I.T.[1]	392 KeV(64.9%)

[1] I.T. = Internal Conversion

[2] E.C. = Electron Capture

Table 2. Characteristics of Some Radionuclides Used for Radioimmunotherapy

Radioisotope	Half-Life	Decay mode	Major decay energy (E-max)
I-131	8.05 d	β^-	0.608 MeV(86%)
Y-90	64 hr	β^-	0.279 MeV(100%)
Cu-67	62 hr	β^-	0.091 MeV(7%)
			0.093 MeV(17%)
			0.184 MeV(49%)
Bi-217	1 hr	β^-	8.78 MeV
At-211	7.2 hr	α	5.90 MeV(41%)
		E.C.[1]	
P-32	14.29 days	β^-	1.71 MeV(100%)
Re-188	16.98 hr	β^-	2.12 MeV(100%)
Sc-47	3.4 days	β^-	0.60 MeV(100%)

[1] E.C. = Electron Capture

Table 3. Radioimmunodetection of Human Cancers with Radiolabelled Antibodies

Mab	Antigen	Radiolabel (Method)	Cancer Type Detected	Comments	Reference
B72.3	HMT[1] -TAG[2] 72	I-131 (Iodogen)	Colorectal carcinoma	- 70%(99/142) of patients have tumor radiolocalization index >3. - 17%(24/142) of patients have tumor radiolocalization index >10. - mucinous adenocarcinomas have the highest radiolocalization index.	27
250-30.6	HCC[3] -HT-29	I-131 (Cl-T)	Colon adenocarcinoma	- 48 hour scans and background substraction gave the clearest picture. - considerable amount of uptake in the liver. - 90% success rate.	28,29
F023C5	CEA[4]	I-131 (Iodogen) In-111 (DTPAan)	Colorectal carcinoma	- no correleation between serum CEA level and immunoscintigraphy results. - 77% success rate. - 25% positive when metastases localization to liver. - peak of tumor to background ratio reached within 48-96 hours. - no difference between I-131 and In-111 radiolabel.	30

Continued...

17-1A	HCC[3]	I-131 (Iodogen)	Colorectal carcinoma	-	Background substraction used.	31,32
				-	best images at 6 to 7 days for whole MAb.	
				-	for $F(ab')_2$ fragments, best images at 4 to 5 days.	
VIT-23e	CEA[4] I-131		Colorectal and medullary thyroid carcinoma	-	transaxial tomoscintigraphy used.	33
				-	16 out of 17 tumor sites detected.	
α-CEA	CEA[4]	I-131 (ICl) In-111 (DTPAan)	Colorectal carcinoma	-	In-111 produced a better signal to noise ratio in the scans.	34, 35
				-	In-111 labelled Ab continue to accumulate in the tumor areas longer than I-131 labelled.	
PK1G	CEA[4]	I-131 (Cl-T)	Colorectal carcinoma	-	liposomally entrapped second antibody.	36
				-	3 out of 5 imaged successfully.	
				-	image at 2, 4, 24, 48 hours.	
AUA1	PLAP[5]	I-125, I-131 (Iodogen)	Colorectal ,ovarian and breast carcinoma.	-	immunohistochemical staining and radiolabelled MAb binding to tumor.	37
				-	a maximun localization index of 134.0(tumor to blood) detected at 3 days post-administration in a patient with colonic cancer.	

Continued...

19.9	HCC[3] -CA 19.9	I-131 (Iodogen) In-111 (DTPAan)	Colorectal and pancreatic carcinoma	For I-131 labelled Ab - background substraction used. - $F(ab')_2$ fragments showed significant accumulations in 66% of cases. For In-111 labelled Ab - 67% success rate, image at 48-72h - only significant source of label instability is transcomplexation to transferrin.	38,39
BI4.011	CEA[4]	I-131 (Iodogen)	Colorectal carcinoma	- 55% success rate for $F(ab')_2$. - optimal time for imaging 3-4 days post injection. - no tumor site identified with intact MAb.	40
H17E2	PLAP[5]	In-111 (DTPAan)	Ovarian, cervical testicular carcinoma	- no background subtraction is required. - high liver uptake. - images better than ultrasound, CT.	41
NDOG$_2$	PLAP[5]	I-123 (Iodogen)	Ovarian carcinoma	- mean plasma clearance time was 20.8 hours. - tumor to background ratio from 1.4 to 4.8 at 3.0 to 20 hours post admininstration.	42
HMFG2	HMFGM[6]	I-123, I-125, I-131 (N-bromo succinimide)	Ovarian carcimoma	- higher levels of radioactivity were seen in blood, normal tissue and solid tumor after iv infusion than ip injection. - absolute uptake in tumor is small. - minimal uptake in solid tumor masses.	37, 43

Continued...

αH-HCG	HCG[7]	I-131 (Cl-T)	testicular carcinoma	-	background substraction required. - image at 24 and 48 hours. - reveal sites not found with CT or ultrasound.	44
PAY 276	HPAP[8]	In-111 (DTPAan)	Prostatic carcinoma	-	75%(19/25) success rate - up to 75.4% of metastases show up with dose up to 80mg.	45
p 96.5	HM[9] -p97	I-131 (Iodogen) In-111 (DTPAan)	Malignant melanoma	-	background substraction required for I-131. - F(ab) fragments superior to intact IgG. - increase F(ab) dose improved image quality. - In-111 uptake in liver noted. - pre-infusion with unlabelled p96.5 decreased the liver/heart ratio with In-111.	46-50
48.7	HM[9] -HMWA[10]	I-131 (Cl-T)	Malignant melanoma	-	background substraction required. - image at 48 hours. - use for imaging and therapy.	49, 51
225.28S	HM[9] -HMWA[10]	I-123 (Iodogen) In-111 (DTPAan) Tc-99m (DTPAan)	malignant melanoma	-	Background subtraction for I-123. - F(ab) superior to IgG.	53, 54

Continued...

9.2.27	HM[9] -HMW cell surface antigen	In-111 (DTPAan)	Metastatic melonoma	-	increased MAb dose, decreased spleen and liver uptake, and enhanced image quality.	55
				-	MAb dose may be a critical determinant of biodistribution and tumor uptake.	
ZME-018	MAA[11] -Gp 240	In-111 (DTPAan)	Metastatic melanoma	-	78% (14/18) success rate.	56, 57
				-	increased dose, increased number of positive scan.	
				-	increased T-1/2 in blood with increased MAb dose.	
LON-M8	HMFG[4] membrane	I-123, I-125, I-131 (Iodogen) In-111 (DTPAan)	Breast carcinoma	-	In-111 uptake in liver.	58
				-	I-MAb do not detect bone or soft tissue metastases.	
				-	In-111 MAb detect bone but not soft tissue metastases.	
3E1.2	HBC[12]	I-131 (Cl-T)	Breast carcinoma	-	administrated s.c.	27
				-	positive on affected lymph nodes.	
791t/36	HOS[13] -179T	I-131 (Iodogen) In-111 (DTPAan)	Bone maligniancies	-	background substraction required for I-131.	59 - 62
				-	87% success rate.	
				-	In-111 comparable to I-131.	
				-	increase uptake of In-111 in both the tumor and normal tissue.	
				-	image at 48 to 72 hours.	

Continued...

LGL-1D6	HG[14]	I-131 (Cl-T)	Frontal lobe glioma	- MAb from β-lymphocyte of glioma patient. - potential as targeting agent for chelated and cytotoxic agent.	63
AF01	AFP[15]	I-131 (Iodogen)	Hepatocellular carcinoma	- 59% (10/17) AFP secreting HCC were visualized. - improved by Fab fragments - immunotomoscintigraphy instead of planar scintigraphy.	63
T 101	CTCL[16] - T65 glycoprotein	I-131 (Cl-T) In-111 (DTPAan)	Cutaneous T-Cell Lymphoma	For I-131 - t 1/2 = 27 hours - clearance through kidneys, free I-131 detected in urine. For In-111 - prolonged retention of radioactivity in skin tumors, erythroderma and lymph nodes. - t 1/2 > 7 days. - sc injection, MAb binding in nodes nearest to injection site depending on MAb dose. - optimal sc dose 0.5mg.	64, 65

List of Abbreviations
1. HMT - Human mammary tumor
2. TAG - Tumor associated antigen
3. HCC - Human colorectal carcinoma
4. CEA - Carcionembryonic antigen
5. PLAP - Placental alkaline phosphatase

6. HMFGM - Human milk fat globule membrane
7. HCG - Human chorionic gonadotropin
8. HPAP - Human prostatic acid phosphatase
9. HM - Human melanoma
10. HMWA - High molecular weight antigen
11. MAA - Metastatic melanoma
12. HBC - Human breast carcinoma
13. HOS - Human oseteogenic sarcoma
14. HG - Human glioma
15. AFP - Alphafetoprotein
16. CTCL - Cutaneous T-cell Lymphoma

usually absent or occur in only minute amounts in normal tissues. The phenotypic expression of a tumor associated marker varies from neoplastic cell to cell, and a variation in production of the markers exists during the natural progression of the malignancy[66,67]. Thus, it may be possible to correlate the level of tumor markers to the tumor burden, although in some cases the tumor may lose its ability to produce the marker during the course of therapy[67].

Antibodies raised against tumor associated markers have the potential for use in tumor localization and other clinical applications[61,69]. The utility of various tumor-associated markers in clinical practice has recently been reviewed by Zalcberg and McKenzie[69]. For radioimmunodetection of malignancy, the ideal tumor associated marker should be expressed on the surface of the tumor and be immunologically detectable, to allow the *in vivo* interaction with the radiolabelled tracers. To enable adequate detection, the tumor associated marker should also be expressed during all stages of the cell cycle, and upon binding with radiolabelled tracer, should not be shed very rapidly[66].

Carbohydrate containing antigens constitute an important type of tumor associated markers. Carbohydrate chains can be found on the surface of cells, attached to glycoproteins and glycolipids and forming a glycocalyx over the cell membrane[70,71]. They are known to participate in important cell-cell interactions and in the regulation of cell growth and differentiation, and may play an important role in maintaining the malignant phenotype[70,73]. During oncogenic transformation the structures of these cell surface carbohydrate chains may be changed as a result of altered levels or activity of one or more glycosyltransferases[72]. One example of a cell surface carbohydrate antigen is the Thomsen-Friedenreich antigen which normally occurs in a cryptic form in normal tissues, but in an exposed form in many malignanices.

2.2.1 The Thomsen-Friedenreich Antigen

In 1928, Thomsen and Friedenreich discovered that bacterial contamination in red blood cells uncovered a normally cryptic receptor on the erythrocyte and rendered the red

blood cell agglutinatable by all human sera including one's own[74]. The causative agent was found to be a bacterial enzyme now known to be neuraminidase[75]. This enzyme is also termed Receptor Destroying Enzyme (RDE) or sialidase[76,77]. The action of this enzyme involves the removal of the terminal sialic acid residues (N-acetyl neuraminic acid) and reveals the normally cryptic antigen, which is named the Thomsen-Friedenreich antigen (T-antigen). It has been further demonstrated that the expression of cryptic T-antigen occurs not only on red blood cells from human and several animal species but also on liver, muscle and most strongly on brain tissue[77].

2.2.1.1 The Composition of the Thomsen-Friendenreich Antigen

Neuraminidase treatment or mild acid hydrolysis of MN glycoproteins on red blood cells will uncover the T-antigen with concomitant release of neuraminic acid[78,79]. The properties of the T-antigen prepared from human red blood cells are listed on table 4.

The Thomsen-Friendenreich antigen can be defined as belonging to the glycophorin type of erythrocyte membrane glycoprotein and is located near or on the amino terminal area[80]. The composition of the glycoprotein may vary from species to species and even from M to N specificity[80]. The alkali-labile carbohydrate part of the T-antigen is represented by the T-disaccharide structure, β-D-galactosyl-α-(1→3) N-acetyl-D-galactosamine, and is common to all T-receptors. This carbohydrate unit is also detected by a plant lectin, peanut lectin *(Arachis hypogaea)*[81,82].

2.2.1.2 M, N, T and Tn Antigens

The second human blood group system, the MN system, was discovered by Landsteiner and Levine[83]. It was shown by Springer *et al*[84,85] that the blood group MN antigenic determinants are not the products of allelomorphic genes, but that N is the precursor substance of M, and the immediate biosynthetic precursor of the MN blood group system are the T and Tn antigen. Their biochemical relationship is shown in

Table 4. Physical and Chemical Characteristics of the Thomsen-Friedenreich Antigen from Human Red Blood Cells[71, 79]

A. Physical Characteristics:

Sedimentation constant	11.1s
Diffusion constant	1.62F
Partial specific volume	0.696 ml/g
Molecular weight	555,000 daltons

B. Carbohydrates (wt%)

Galactose	13.21
Mannose	6.43
Fucose	0.83
N-Acetylgalactosamine	10.27
N-Acetylglucosamine	7.37

C. Amino Acids (wt%)

Aspartic acid	3.49	Isoleucine	3.00
Threonine	5.07	Leucine	4.84
Serine	4.26	Tyrosine	2.12
Glutamic acid	5.01	Phenylalanine	2.20
Proline	3.49	Lysine	2.75
Glycine	1.74	Histidine	2.48
Alanine	2.63	Arginine	3.02
Half cystine	0.00	Methionine	2.93
Valine	3.65	Tryptophan	0.00

Figure 1. The Biochemical Relationship Between MN, T and Tn Antigen.

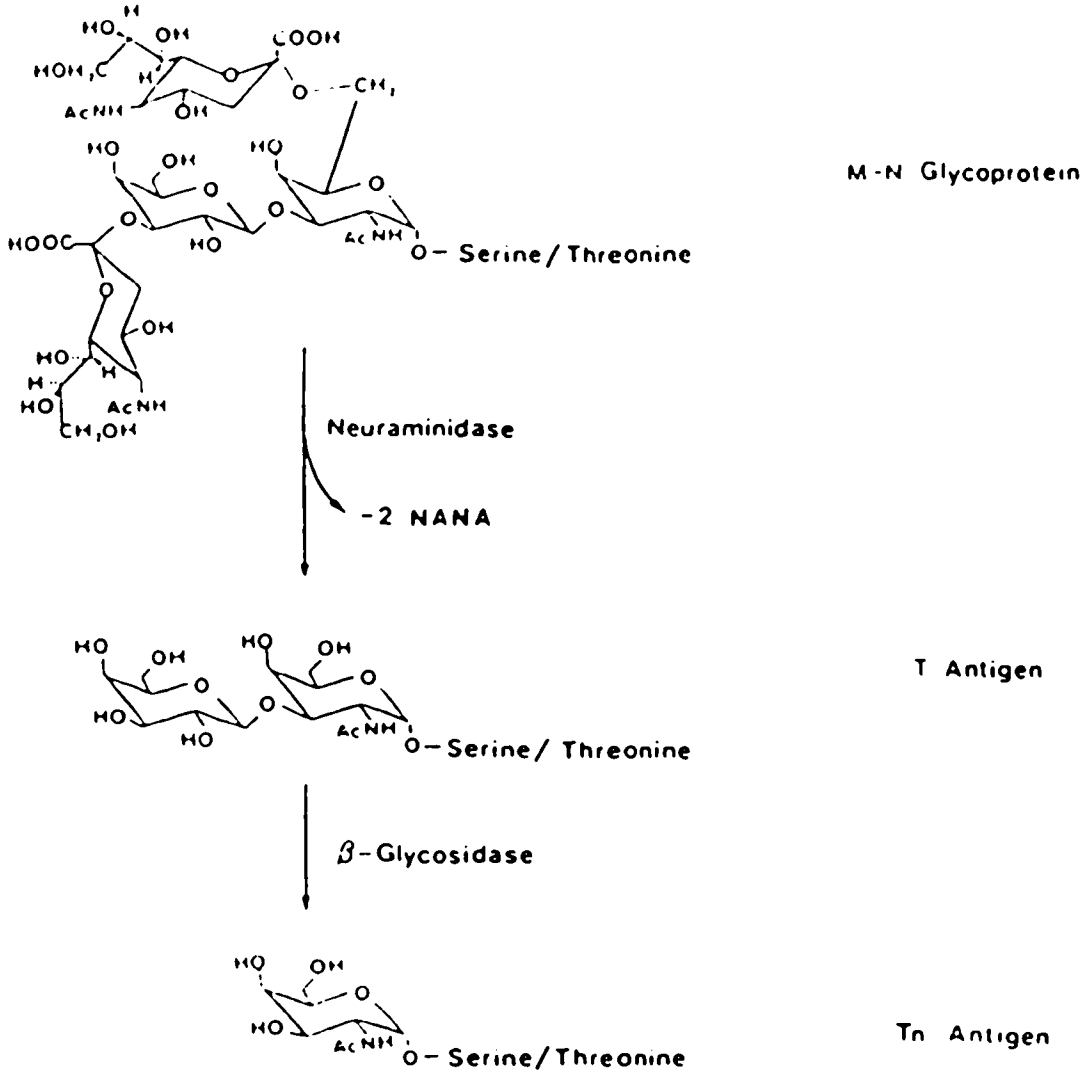


figure 1[77].

Human red cell M and N antigens are aggregates of sialoglycoproteins, composed of identical subunits[77,84]. The molecular weight of the aggregates range from $3x10^4$ to at least $1.2x10^7$ depending upon the method of isolation[87]. M and N antigens differ from each other in the terminal immunodominant carbohydrate structure[84,91]. The peptides that carry the M and N specific structures on erythrocytes differ from one another in two amino acids; the amino terminus for M is serine, while it is leucine for N, and the amino acid fifth from the amino terminus is glycine for M and glutamic acid for N[77]. Terminal N-acetylneuraminic acid (NANA) is an essential component of both M and N specificities since the sole removal of NANA leads to inactivation of both antigens[81].

Neuraminidase treatment or mild acid hydrolysis of MN glycoproteins uncovers the T-antigen with a concomitant release of NANA exclusively. β-D-galactosidase treatment of T-antigen uncovers the Tn antigen[77,89]. Covered T and Tn specific structures also occur in carbohydrates that do not terminate in the M and N immunodeterminants[90,91].

T antigenic receptors in healthy human tissues are shielded by O-glycosidically linked carbohydrates, but immunoreactive T-antigen is present in the cytoplasm and on the outer cell membrane of approximately 90% of the major forms of carcinoma and T lymphoma, as determined by absorption of human anti-T antibodies and immunohistochemistry[84,85]. The major cases of immunoreactive Tn antigen are believed to be the result of spontaneous mutation and frequently associated with *in vivo* thrombocytopenia and leukemia[92]. Tn antigen is also detected in primary breast carcinoma and their metastases[84]. This makes T and Tn antigens important tools in the study of carcinoma cell surface antigens and the host reponse to them.

2.2.1.3 The Tumor Specificity of T-Antigen

The observation that the T-antigen is expressed on many human carcinoma cells, but is masked by sialic acid on homologous normal epithelia suggests that the T-antigen is

a tumor associated antigen[94,95]. Inhibition of agglutination of erythrocytes bearing the T-antigen by anti-T antisera or peanut lectin with extracts of human carcinomas reveals the presence of T-antigen active substances in the carcinomas[93]. The T-antigen could be detected in homologous normal tissues only after neuraminidase treatment[93].

Several mechanisms have been proposed for the presence of non-cryptic T-antigen on tumors. It may be due to the alteration of specific glycosyl transferases in malignancies which block the incorporation of sugar residues at or near the non-reducing terminus of oligosaccharide chains[96], or the inability of tumors to synthesize certain classes of glycoconjugates due to the lack of the necessary substrates[93]. Another possiblity is the enzymatic removal of the cell surface glycoconjugates by highly active tumor enzymes such as glycosidases or proteases, and thus unmasking the cryptic T-structure on the tumor cell surface[91].

The relative proportions of T-antigens on human carcinomas frequently correlate with the carcinomas' aggressiveness. The majority of low grade tumors are T-antigen negative or cryptic T-antigen positive, while high grade or highly invasive tumors are usually T-antigen positive[96,97]. It is also found that tumor cell adhesion to hepatocytes can be specifically inhibited by T and Tn specific glycoconjugates, suggesting that they may be involved in specific cell-cell adhesions required for invasion and metastasis by cancer cells[94,95].

In normal healthy mammalian tissues, the T-antigen is masked by sialic acid residues in many common membrane glycoproteins and glycolipids. However, many animal and human tumors have been reported to express the T-antigen in a non-cryptic form, which can be detected by both chemical and serological methods[98-103]. In animals, the TA_3 spontaneous mouse mammary adenocarinoma[98,99], the RI lymphoma[100,101] and the ASPG-1 rat mammary adenocarcinoma[102,103] have been shown to express the non-cryptic T-antigen.

The TA_3/Ha murine mammary adenocarcinoma animal model which was employed in the experimental procedures of this thesis project has been well characterized as to the T-antigen expression. This tumor line has been shown to bind PNA both *in vitro* and *in vivo* [104]. Epiglycanin, a high molecular weight (500,000 dalton) glycoprotein known to contain the immunodeterminant structures of the T and MN antigens, is present on the TA_3/Ha tumor cell surface[99]. Epiglycanin is composed largely of N-acetyl-galactosamine, galactose, sialic acid, serine and threonine[99]. Epiglycanin on the TA_3/Ha tumor cell surface is shed into the serum and may be responsible for the transplantability of the TA_3/Ha cell line[98]. Specific lung or liver metastatic variants of the TA_3/Ha cell line have been selected and employed as animal models for the study of localization of radioiodinated PNA[104].

The TA_3/St subline is a spontaneous variant of the TA_3/Ha but it does not secrete epiglycanin and lacks the ability to grow in a host other than the strain of origin (strain A mice)[97]. PNA binding in both tumor lines has been well documented[104] and thus it is a convenient model to use for the comparison of the localization of PNA labelled with different isotopes.

2.2.1.4 T-antigen Expression on Human Tumors

T-antigen has been termed a "tumor associated antigen" since it is present in an unmasked form in many cases of cancers of epithelial and possible neuroectodermal origin but not on corresponding healthy tissues[84,95]. A large variety of malignant, benign and healthy tissues, as well as cell cultures are examined by various immunoreactive methods to check for the presence of the T-antigen. These methods include immunohistochemical staining of tissues sections with PNA, polyclonal or monoclonal anti-T antibodies, measurement of decrease of *in vivo* anti-T titre, and the presence of delayed hypersensitive reaction to injected purified T-antigen[81,94,95]. So far, the occurance of T-antigen has been demonstrated on malignant tumors of the human breast[106-108], urinary bladder carcinoma[109-111], lung adenocarcinoma[107], colon and gastric carcinomata[112,113] and

in some T cell lymphomas and leukemias[84]. The exposed T-antigen has not been, so far, found in sarcomas, benign tissues, thyroid carcinoma or tissues with non-cancerous disease[114-116].

In various studies, T-antigen was found in all 45 colon and 25 of 26 breast carcinomas examined[85]. Springer *et al* [85] found T-antigen in 95 percent of 144 fresh surgical samples of all types of primary carcinomas from all major organs, and on the outer cell membranes of 25 of 26 human breast carcinoma derived epithelial cell lines.

It has also been shown that in urinary bladder cell carcinoma, most low grade tumors were usually cryptic T-antigen positive, whereas high grade tumors were almost all T-antigen positive or cryptic T-antigen negative[109,110]. This information can be used as a predictor of biological behaviour in tumors that are histologically indistinguishable. Springer *et al* [85,106] suggested that for some forms of carcinoma, the densities of T and Tn antigen on the carcinoma cells are histochemical predictors of the carcinoma's invasiveness and when present in the primary tumor, T and Tn antigens were found in all metastases, as well as in tissue cell cultures derived therefrom, which points to the clonal nature of the antigen[84]. They have also shown that *in vitro* clusters of T and Tn epitopes are involved in the initial adhesion of some cancer cells to healthy epithelial tissues.

Orntoft *et al* [113] suggest that the T-antigen may occur early in the malignant transformations in human colorectal cancer and could be of potential value in the identification of patients at risk.

2.2.1.5 Anti-T Antibodies

All humans and most animals are reported to possess anti-T antibodies *in vivo* due to the continuous antigenic stimulation by their own intestinal flora[84,117]. Numerous symbiotic gram negative bacteria isolated from the stools of healthy individuals presented the T-specific antigen[117]. Under prolonged oral administration of antibiotic, many individuals will experience a decrease in anti-T antibodies levels due to the diminished gastrointestinal flora[87].

Human anti-T antibodies consist predominally of IgM, constituting 7-14% of total IgM, with some IgG and very little IgA[106]. The T-antigenic structure is recognized by the immune system as foreign, and cancer patients have been found to show a strong respond to the T-antigen on their tumor cells, although the reaction may not be clinically significant[106].

Anti-T agglutinin levels have been found to remain quite steady in a given healthy adult. However, severely depressed anti-T scores have been observed to be associated with a number of breast, respiratory or gastrointestinal tract carcinomas[84,85]. It has been suggested that the depression of anti-T titre levels is due to the interaction of anti-T antibodies with T-specific antigens or closely related glycoproteins and possibly glycolipids which are either attached to the cancer cell surface and/or released into the circulation[85]. Surgical removal of the primary tumor led to a rebound or overshoot of anti-T titre score. This rebound implicates the carcinoma as the cause of depressed anti-T antibodies rather than a genetic defect that would render the patient unable to produce anti-T antibodies[106].

The titrations of anti-T antibodies are usually done by routine blood banking procedures. In the assessment of anti-T titre scores, the hemagglutination method is specific but not sensitive enough. Therefore, a solid-phase immunoassay for the quantitative measurement of anti-T immunoglobulins and total serum immunoglobulins has been developed[106]. From this assay, it is found that in carcinoma patients, only anti-T IgM levels are different from those of other patients and healthy persons. The detection of carcinoma was most sensitive when a person's anti-T IgM was related to total serum IgM by establishing the value Q by the formula $[(\text{anti-T IgM})^2/\text{total IgM}]\times 100$ [106]. In 183 carcinoma patients tested, 164 had a Q value outside the normal range, giving a sensitivity of 89.6 percent[106]. Thus this assay may be useful in screening and for early detection of carcinoma.

2.2.1.6 Cellular Immune Response to T-antigen

The cellular immune response to T-antigen can be measured both *in vivo* and *in vitro*. The leukocyte inhibition test can be used to determine cellular immunity to the T-antigen *in vitro*, while the *in vivo* response is measured with the delayed type hypersensitivity reaction to T-antigen (DTHR-T) following intradermal injection[84,85,106].

Table 5 summarizes the results after a single injection of T-antigen to various groups of subjects. The DTHR-T reaction detected 112 of 124 patients with adenocarcinoma, squamous-cell carcinoma, or small-cell carcinoma in body cavities, while out of the 85 healthy individual tested, none showed a positive DTHR-T reaction[85].

Measurement of cellular immune responses *in vitro* oversimplifies conditions seen *in vivo*. Researchers found the *in vitro* responses to carcinoma associated T-antigen to be less sensitive and less specific than the DTHR-T[106]. *In vitro* responses to the T-antigen's defined cleavage products, which carry hapten clusters on their peptide chains, are more sensitive than those towards the intact T-antigen or the synthetic T-haptenic disaccharide[106].

Another class of compounds which are useful in the study of cell surface carbohydrates are lectins. Peanut Agglutinin (PNA) is widely used in the immunodetection of the T-antigenic structure since it has an affinity for the immunodeterminant group of the T-antigen. Therefore, PNA, since it is used as an immunohistological stain in certain T-antigen expressing animal and human tumors, has also been investigated as a tumor localizing agent.

2.3 Lectins

In 1888, Stillmark obtained a partially purified proteinaceous preparation from castor beans that he called ricin. He observed that upon addition of ricin to red blood cells, the cells stuck together "like in clotting"[118]. Thus the term "hemagglutinin" was introduced for the first time by Elfstand (1898) for plant proteins that cause clumping of cells, due to the

Table 5. Intradermal delayed-type Hypersensitivity Response to T-antigen, of Carcinoma Patients and Controls[85]

Category	DTHR-T+/total tested
Lung	
Carcinoma	
Adeno	45/59
Bronchioloaveolar	5/6
Small-cell	15/17
Squamous-cell	12/14
Large-cell anaplastic	1/1
Other pleuropulmonary cancers	1/5
Benign diseases	2/35
Pancreas	
Adenocarcinoma	23/26
Pancreatitis	0/13
Breast adenocarcinoma	
Ductal	
Stage I noninfiltrating	10/12
Stage I infiltrating	32/37
Stage II and III	37/42
Stage IV	17/17
Lobular	
Stage I noninfiltrating	7/10
Stage I infiltrating, II and III	7/19
Tubular	1/4
Benign breast disease	11/144
Urinary bladder	
Transitional-cell carcinoma	19/23
Cancers originating elsewhere	
Adenocarcinoma	11/11
Melanocarcinoma	3/5
Other cancers	5/24
Non-cancer diseases not related to lung, pancreas, or breast	
Tumors	0/14
Other diseases	0/40
Healthy	0/85

similarity of their activity to that of human and animal serum agglutinins[119].

Later, several of this group of "hemagglutinins" were found to be blood group specific, and Boyd introduced the name "lectin", from the Latin, "to pick out or to choose", for such a group of compounds[120]. The subsequent discovery of many similar substances in both animal as well as plant tissues has prompted several attempts to reach a common agreement as to how to define the term "lectin". Gold and Phelps[121] used the term "receptor-specific proteins" to cover the wide range of reagents found in viruses, bacteria, fungi, plants, snails, fish and other lower vertebrates. The broader adjective "receptor specific" was chosen rather than the narrower "sugar-specific" used by Sharon and Lis[122] since at that time a small number of lectins were already known which were not inhibited in hemagglutination tests by simple sugars or oligosaccharides.

The definition for lectin most commonly adopted is the one proposed by Goldstein *et al* [123] . They suggested lectin as a carbohydrate-binding protein of nonimmune origin that agglutinates cells or precipitates polysaccharides or glycoconjugates. This definition is adopted by the Nomenclature Committee of the International Union of Biochemistry. The definition implies that lectins are multivalent, that is, they posses at least two sugar binding sites which enable them to agglutinate animal and plant cells and/or to precipitate polysaccharide glycoproteins, glycolipids, etc. The emphasis on "nonimmune" is included in the definition in order to distinguish lectins from anti-carbohydrate antibodies which may act as cell agglutinins[123].

However, Goldstein's definition of lectin has received criticism, since if it is rigorously applied, it will exclude sugar-binding proteins with a single binding site. Kocourek *et al* [124] (1981) suggested that the emphasis should be put on the sugar-binding properties and the lack of enzymatic activity towards carbohydrate structures, whereas the number of sugar-binding sites or agglutinations/precipitation activities should not be considered.

2.3.1 Detection of Lectins

Traditionally, based on their ability to agglutinate erythrocytes, lectins are detected by hemagglutination using a panel of freshly drawn animal or human erythrocytes , or red blood cells (RBC) treated by neuraminidase, papain or trypsin, which are more sensitive to agglutination[125,126]. More refined screening procedures are based on the ability of these carbohydrate-binding proteins to precipitate glycoconjugates and polysaccharides. Formation of a precipitate between a lectin and carbohydrate-containing macromolecules, is indicative of lectin activity.

Affinity electrophoresis, a technique that combines the principles of affinity chromatography and electrophoresis was devised by Horejsi and Kocourek[127] for the detection of lectins. Proteins are subjected to electrophoresis on a matrix formed by copolymerization of alkenyl glycosides with acrylamide and the lectins having sites complementary to the ligand are retained, while other proteins undergo a normal separation.

Two recent procedures for the detection and preliminary characterization of lectins involve (a) using polystyrene particles containing adsorbed glycoconjugates to test for agglutination from plant extracts[128], and (b) assaying for the adherence of erythrocytes to the lectin-coated wells of polystyrene microliter plates[129].

2.3.2 Isolation and Physiochemical Properties

Isolation of lectins generally begins with a saline (or buffer) extraction of the finely ground seed meal. Purification of the lectin usually involves the use of affinity chromatography which utilizes the specific sugar-binding capacity of the lectin to separate it from other proteinaceous material. The application of affinity chromatography as a technique for lectin purification has been extensively reviewed[130-132].

Lectins occur in a wide range of plants, bacteria and vertebrates, and the lectins purified from these sources show diverse physical and chemical characteristics. There are no common structural features except that they are protein in nature[133]. Many of the lectins

found have been determined to be glycoprotein, but there are several notable lectins such as the peanut lectin, concanavalin A and wheat germ lectin that lack any covalently linked sugar[130]. Many lectins are found to be composed of several subunits, usually of equal or similar molecular weight although some are made up of light and heavy chains[134,135]. The number of subunits may vary from lectin to lectin, but the most common number seems to be four, with a typical molecular weight of 120,000 (4x30,000). In general, the molecular weight of lectins have been found to range from 36,000 to 335,000 [135]. Another interesting feature of lectins is that some of them may require certain metal ions such as Mn^{2+} and/or Ca^{2+} for their reactivity[133].

2.3.3 Carbohydrate-Binding Specificity of Lectins

Lectins interact with oligosaccharides of cells or glycoprotein surfaces. Consequently, free oligo- or mono-saccharides of the appropriate specificity are able to inhibit or even reverse this interaction. The specificity of the lectin is considered to be rather "relative" since it is usually represented by the sequence of carbohydrates with the best inhibitory effect on the lectin-induced agglutination or precipitation reaction known. Other carbohydrates inhibitors may also be available although the inhibition effects are not as pronounced[136].

It is important to establish the carbohydrate-binding specificity of a lectin in order that it may become a useful tool in biochemical or immunochemical studies. Most plant and animal lectins may be classified into a rather limited number of carbohydrate-binding groups, although within the same group, their detailed sugar specificity may be different[137]. These carbohydrate-binding groups include the mannose/glucose binding lectins, the N-acetylgalactosamine/galactose binding lectins, the N-acetylglucosamine binding lectins, the L-fucose binding lectins, sialic acid binding lectins, and those with "complex" binding sites[137]. Examples of some of the more common lectins in each group and their sugar binding specificities are summarized in table 6.

Table 6. Saccharide-Binding Specificities of Lectins[137]

Lectin	Nominal Specificity
A. GLUCOSE/MANNOSE BINDING LECTINS	
Canavalia ensiformis (Jack Bean)	Manα1,2Manα1,2Man> Manα1,2Man> α-Man> α-Glc> α-GlcNAc
Dioclea grandiflora	α-Man> α-Glc
Lathyrus odoratus (Sweet Pea)	α-Man> α-Glc> GlcNAc
Lathyrus sativus (Chickling Vetch)	α-Man> α-Glc
Lathyrus tingitanus (Tangier Pea)	α-Man> α-Glc
Lens culinaris (Lentil)	α-Man> α-Glc, α-GlcNAc
Onobrychis viciifolia (Sainfoin)	α-Man> α-Glc
Pisum sativum (Pea)	α-MAn*p*> α-Glc*p*> α-GlcNAc*p*
Vicia cracca (Common Vetch)	α-Man> α-Glc
Vicia faba (Fava Bean)	α-Man> α-Glc = α-GlcNAc
B. N-ACETYLGLUCOSAMINE-BINDING LECTINS	
Brachypodium sylvaticum (False Brome Grass)	(-GlcNAcβ1,4)*n* > GlcNAc
Datura stramonium (Thorn Apple, Jimsonweed)	GlcNAcβ1,4GlcNAcβ1,4GlcNAcβ1,4GlcNAc > ClcNAcβ1,4GlcNAc >> GlcNAc
Griffonia Bandeiraea simplicifolia Lectin II	α-GlcNAc = β-GlcNAc
Hordeum vulgare (Barley)	(-GlcNAcβ1,4-)*n* >> GlcNAc
Lycopersicon esculentum (Tomato)	GlcNAcβ1,4GlcNAcβ1,4GlcNAcβ1,4GlcNAc > GlcNAcβ1,4GlcNAcβ1,4GlcNAc > GlcNAcβ1,4GlcNAc
Oryza sativa (Rice)	GlcNAcβ1,4GlcNAcβ1,4GlcNAc > GlcNAcβ1,4GlcNAc >> GlcNAc
Phytolacca americana (Pokeweed, Pigeon Berry)	(GlcNAcβ1,4GlcNAc)$_{1-3}$
Solanum tuberosum (Potato)	GlcNAc(β1,4GlcNAc)$_2\beta$1,4GlcNAc > GlcNAcβ1,4GlcNAcβ1,4GlcNAc > GlcNAcβ1,4GlcNAc >> GlcNAc
Triticum vulgare (Wheat; Wheat Germ Agglutinin)	GlcNAcβ1,4GlcNAcβ1,4GlcNAc > GlcNAcβ1,4GlcNAc >> GlcNAc
Ulex europaeus (gorse or Furze Seed) Lectin II	L-Fucα1,2Galβ1,4GlcNAc = L-Fucα1,2Galβ1,4Glc > GlcNAc(β1,4GlcNAc)$_1$1,4GlcNAc > GlcNAcβ1,4GlcNAcβ1,4GlcNAc = GlcNAcβGlcNAc
C. N-ACETYLGALACTOSAMINE/GALACTOSE-BINDING LECTINS	
Phaseolus lunatus (Lima Bean)	GalNAcα1,3?L-Fucα1,2?Gal > α-GalNAc > α-Gal
Amphicarpaea bracteata (Hog-Peanut)	GalNAcα1,3GalNAc > GalNAcα1,3Gal > α-GAlNAc
Dolichos biflorus (Horse Gram)	GalNAcα1,3GAlNAc > α-GalNAc > α-Gal
Helix pomatia (Edible Snail)	GalNAcα1,3GalNAc > α-GalNAc > α-GlcNAc > α-Gal
Vicia villosa (Hairy Vetch)	α-GalNAc > α-Gal
Wistaria floribunda Agglutinin	GalNAc > Gal
Sophora japonica (Japanese Pagoda Tree)	GalNAc > Gal
Glycine max (Soybean)	α-GalNAc = β-GalNAc > α-Gal
Griffonia (Bandeiraea) simplicifolia (Lectin I, GS I)	GS I-B$_4$, α-Gal > α-GalNAc; GS I-A$_4$, α-GalNAc > α-Gal

Arachis hypogaea (Peanut)	Galβ1,3GalNAc > $GalNH_2$ > Gal
Vicia graminea	(Galβ1,3GalNAcα1-O-)*n*
Phaseolus vulgaris (Red Kidney Bean)	Galβ1,4GlcNAcβ1→6 Man Galβ1,4GlcNAcβ1→2
Ricinus communis (Castor Bean)	Gal > GalNAc
Abrus precatorius (Jequirity Bean)	Gal > GalNAc
D, FUCOSE-BINDING LECTINS	
Lotus tetragonolobus (Asparagus Pea)	α-L-Fucose
Ulex europaeus (Gorse or Furze Seed) (Lectin I)	α-L-Fucose
Anguilla (Eel) Lectins	α-L-Fucose, 3-O-Methyl-D-Fucose, 3-O-Methylgalactose
Griffonia simplicifolia Lectin IV	L-Fucα1,2Galβ1,3?L-Fucα1,4?GlcNAc
E, SIALIC ACID-BINDING LECTINS	
Carcinoscorpius rotunda cauda (Indian Horseshoe Crab)	Neu5Acα2,6GalOH > Neu5Acα2,6Galβ1,4Glc
Limax flavus (Slug)	Neu5Ac > Neu5Gc
Limulus polyphemus (Horseshoe Crab)	Neu5Gcα2,3GalNAc > Neu5Acα2,6GalNAc > Neu5Ac = Neu5Gc

With few exceptions, lectins interact with the nonreducing, terminal, glycosyl group of polysaccharide and glycoprotein chain ends. With respect to the structural requirements of the sugar residues, many lectins tolerate some variation at C-2 of the sugars that they bind[137]. For example, concanavalin A and the lectins from the pea (*Pisum sativum*), lentil (*Lens culinaris*) and fava bean (*Vicia faba*) all exhibit a primary specificitity for mannose, but will also bind glucose and to a lesser extent, N-acetylglucosamine[138-140]. However, lectins generally tolerate very little variation at C-3 of the sugar that they bind[137]. The C-4 hydroxyl group of carbohydrates (hexopyranose form) is also critially involved in lectin binding. Mannose/glucose binding lectins do not interact with galactose and N-acetylglucosamine binding lectins do not interact with N-acetylgalactosamine[137].

There also exists in some lectins binding sites other than those for carbohydrates and these non-carbohydrate binding sites may have an important role in both lectin function and their application. Concanavalin A and a large series of legume (and some nonleguminous) lectins have been shown to bind fluorescent hydrophobic molecules such as ANS (1,8-anilinonaphthalenesulfonic acid) and TNS (2,6-toluidinylnaphthalenesulfonic acid). A simple high affinity site for adenine and related compounds with cytokinin activity has been identified in the lima bean lectin component III tetramer[141,142].

The nature of the forces involved in carbohydrate-lectin interaction is a subject of considerable interest and some controversy. Based on the polyhydroxylic and hydrophilic nature of sugars, it would be expected that polar interactions such as hydrogen bonding and dipole interactions should play an dominant role in these carbohydrate-protein interactions[143]. X-ray crystallographic studies on concanavalin A - methylα-mannopyranoside complexes and hapten inhibition of precipitation studies support the role of polar interaction in the binding[143]. Lemieux and his colleagues[144-146], however, suggested that the carbohydrate-lectin interaction is hydrophobic in nature, and the oligosaccharide portion of glycoproteins may play a role in the exposure of its hydrophobic residues. Roberts and Goldstein[147] showed that the interaction of L-fucose on the blood group A trisaccharide with lima bean lectins appears

to have hydrophobic characteristics due to the difference in the binding entropy with respect to other sugars. A more conservative hypothesis is that both hydrophilic and hydrophobic forces are involved in the carbohydrate-lectin interactions. The polar interaction may occur between carbohydrate hydroxyl groups and the polar side chains of amino acid residues within a lectin's hydrophobic binding sites[148].

2.3.4 Biological Properties of Lectins

By virtue of their inherent property of specific attachment to carbohydrate moieties, lectins bind readily to the outer carbohydrate coating of cells. Such binding may result in a variety of biological effects and may serve as a basis for their *in vivo* function and the application of lectins to the investigation of chemical and biological problems[149]. Some of the commonly observed biological effects of lectins are outlined below.

2.3.4.1 Agglutination

The most common detectable manifestation of the interaction between lectins and cells is agglutination. The ability to agglutinate cells distinguishes lectins from other sugar-binding macromolecules, such as glycosidases and glycosyltransferases[123]. For agglutination to occur the bound lectin must form multiple crossbridges between the two cells. The agglutination effect is influenced by many factors, such as the molecular properties of the lectin (molecular size, number of sugar binding sites, etc.), cell-surface properties (number and accessibility of receptor sites, membrane fluidity), and metabolic state of the cells[150]. Cells that are not agglutinated by low concentrations of a lectin frequently become agglutinable after very mild proteolysis, although such treatment does not necessarily affect the total number of lectin binding sites[151]. Chemical or enzymatic modifications of lectins, especially those that alter the valency and/or size of the lectin, may have pronounced effect on their biological activites[153]. However, modification of the carbonydrate moieties of lectins that are glycoproteins usually has no effect on their agglutinating activity[152].

2.3.4.2 Mitogenic Stimulation of lymphocytes

Mitogenic stimulation is the triggering of quiescent, non-dividing lymphocytes into a state of growth and proliferation. PHA, the lectin from red kidney bean (*Phaseolus vulgaris*) was the first lectin found to be mitogenic[153]. Since then several other lectins, including pokeweed mitogen and concanavalin A, have also been found to possess mitogenic activity[153]. Many lectins differ markedly in their ability to stimulate lymphocytes of different species, and the mitogenic activity is affected by modifications of the cell surface, as well as of the lectin molecule[135].

In contrast to stimulation by antigen, in which specific clones of lymphocytes are induced to proliferate, lectins activate multiple lymphocyte clones irrespective of their antigen specificity. As a result, the percentage of responding cells is rather high, and up to 70 to 80% of the appropriate lymphocyte population can be affected[154].

The response of the lymphocyte to the mitogenic stimulation by the lectin is depicted in figure 2 [156]. The stimulated lymphocytes, in addition to the metabolic changes, release a variety of biologically active polypeptides, known as lymphokines, such as interleukin 2 (IL-2) and interferon[155].

Klaus and Hawrylowicz[156] suggested that the mitogenic stimulation by a lectin could be separated into two phases. The first phase is mediated *via* a lectin-receptor interaction and represents the transition of the cell from the resting state to G1. This stage is referred to as "competence induction" or "activation". It has been hypothesized that the binding of the lectin to the cell surface leads to cross-linking of receptors, which in turn alters the membrane structure and function. This leads to a generation of a hypothetical signal which triggers a series of biochemical events resulting in cell activation. Another theory suggested that the binding of mitogenic lectin renders the cell "foreign" to the body, and activation of the lymphocytes occur *via* an indirect mechanism.

Figure 2. Events During Mitogenic Stimulation by Lectin [136]

(1) Membrane changes, increased transport.

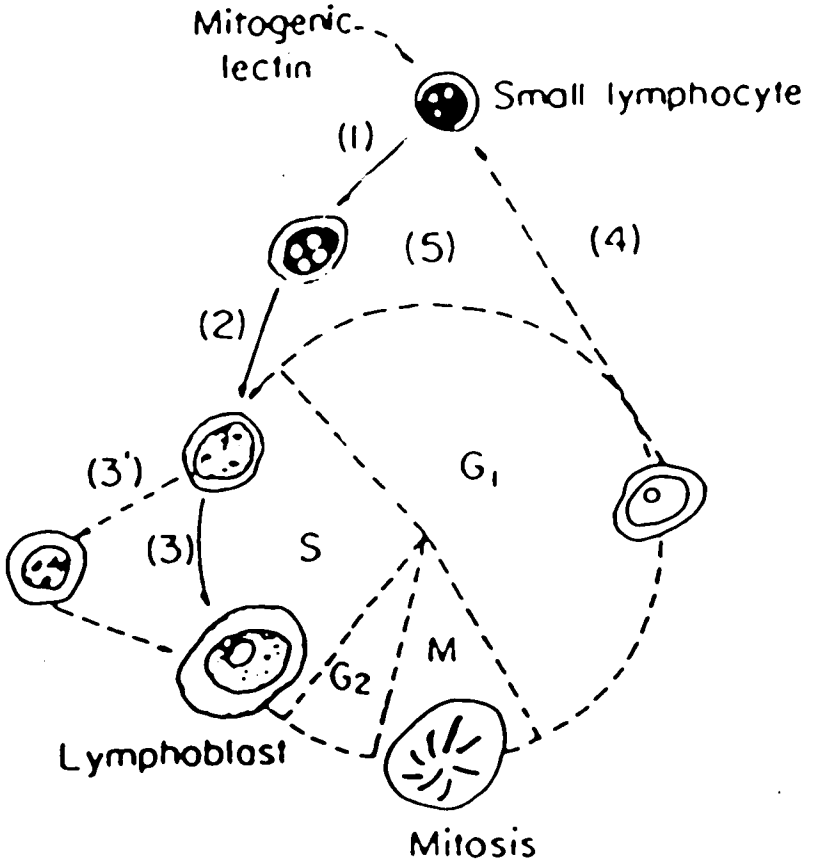
(2) Genetic derepression (RNA, protein synthesis), lympohokine production, increasing cell size, morphological changes.

(3) DNA synthesis, continued lymphokine production.

(3') Differentiation of precursor to effector cell.

(4) Reversion to resting lymphocyte.

(5) Recycle with continued presence of mitogen.



The second, proliferative phase of mitogenic stimulation is initiated by the interaction of interleukin-2 with its specific receptors on the surface of the lympocyte, which leads to DNA synthesis and cell division[156].

2.3.4.3 Toxicity of Lectins

Several lectins, e.g., concanavalin A, wheat germ agglutinin and PHA are toxic to mammalian cells both *in vitro* and *in vivo* [149]. Toxic lectins are generally selective in their action on cells , and transformed cells are usually more sensitive to the cytotoxic effects of lectins than are normal cells[157]. Thus, there have been attempts to try to inhibit tumor growth by lectins *in vivo*. Ricin, concanavalin A and *Griffonia simplicifolia I* lectins have been shown to have a protective effect against tumor growth in experimental animal model[158-160].

2.3.5 Other Biological Effects of Lectins

In addition to agglutination, mitogenic stimulation and cell toxicity, there exists a wide variety of biological effects that are also initiated by the interaction between certain lectins and cells. These include[137,149]:

1. Insulin-like effects on fat cells.
2. Mediation of killing of target cells by lymphocytes and macrophages.
3. Enhancement of phagocytosis of yeasts and bacteria by macrophages.
4. Induction of vacuole formation in macrophages.
5. Promotion of cell adhesion and spreading.
6. Induction of platelet release.
7. Induction of histamine release from basophils and macrophages.
8. Peroxide release from macrophages.
9. Inhibition of endocytosis of lysosomal enzymes by cultured fibroblasts.
10. Inhibition of fungal growth.

2.3.6 Physiological Function of Lectins

Lectins are found in a wide variety of plant and animal species. This widespread occurrence of lectins suggests that they may have some fundamental biological role, especially in plants. Since lectins recognize specific carbohydrate structures, they may play some roles in certain cell recognition events. A variety of functions have been proposed, including plant defense, specific attractants for rhizobical symbiosis, and in the transport, immobilization and storage of carbohydrates[137,149].

One factor that should be considered in the evaluation of lectin's function is the apparent lack of lectins in some plants. Thus, the role of lectins in vital functions of the plant may be questionable[137]. However, it should be recognized that most screening assays for lectins are relatively insensitive and are dependent on the carbohydrate-binding properties of these molecules. Therefore, failure to detect one form of a lectin does not necessarily mean that other forms are not present in the plant[137].

The various hypotheses on lectin functions are briefly summarized in table 7.

2.3.7 Applications of Lectins

The high affinity of lectins for sugar residues and their derivatives, coupled with the possibility of labelling lectins by means of a variety of methods, makes them a powerful tool for use in biochemistry, cell biology, immunology and related areas[137,149].

Some of the current applications of lectins are summarized in table 8 and this list of utilization is growing rapidly.

2.4 Peanut Lectin

The peanut (*Arachis hypogoea*) lectin (PNA) is a readily available plant lectin that possesses anti-T activity. It is also termed as "anti-T agglutinin"[161] due to the similarity between PNA and anti-T antibody of mammalian sera[162]. Peanut lectin is found to be most specific for the carbohydrate structure β-D-galactosyl (1→3) N-acetyl-D-galactosamine[162].

Table 7. Proposed *In Vivo* Functions of Lectins[137]

(I)Plant Lectins

A. Defense mechanism

- may protect plants against pathogens during the imbibition, germination and early growth of the seedings.
- defense against bacteria and viruses.
- defense of plants against animal predators.

B. Pathogenesis

- may aid the invasion by pathogens by serving as receptors for phytotoxin or for attachment of the pathogen.

C. Symbiosis

- lectins may play a role in the binding of the bacteria to the root hairs of the plant.
- lectin recognition of rhizobia may account for the specificity in the initiation of the nitrogen fixing symbiosis.
- lectins may serve as a bridge to cross-link receptors on the rhizobial surface with similiar receptors on the root surface, thus triggering a chain of events leading to effective symboisis.

D. Cell wall elongation

- lectins may serve as a non-covalent glueing substance in the cell wall and play a role in cell wall extension by participating in the breaking and reformation of non-covalent cross-links with other cell wall components.

E. Enzymes

- Hankins and Shannon [144] isolated a hemagglutinin from mung bean seeds that has strong galactosidase activity, however, these may be enzymes with hemagglutinating properties.

F. Others

- may aid in the transport of carbohydrates and their immobilization in the seeds.
- as a storage protein.
- mitogenic stimulation of plant embryonic cells.
- maturation or germination of the seeds.

(II) Animal Lectins

A. Organization of extracellular glycoconjugates.

B. Shuttling soluble glycoproteins from the outside to the interior of the cell and within intracellular compartments.

C. Removal of foreign or unmasked glycoproteins from circulation.

D. Cellular communication and/or adhesion.

Table 8. Applications of Lectins[137].

A. Isolation and Structural Studies of Glycoconjugates.

- detection and identification of carbohydrates by interaction with lectins.
- immunoelectrophoresis with lectin-containing gel to study carbohydrate content and structure.
- affinity chromatography of glycoprotein on lectin-containing columns for purification and isolation.
- fractionation of glycoprotein and glycopeptides which differ only slightly in their structures.

B. Investigation of Complex Carbohydrate Structures.

- study of cell-surface antigens.
- investigation of the architecture of cell surfaces and its change upon malignant transformation.
- localization of lectin-receptors on cell surface or subcellular structures.

C. Separation of Cells

- peanut agglutinin is used to separate murine and human thymocytes into PNA+ and PNA- subpopulations.
- soybean agglutinin separates peripherial blood lymphocytes.
- separation of pluripotential stem cells from alloreactive cells for bone marrow transplant.

D. Mitogenic Stimulation of Lymphocytes.

- study of cell division and the role of lymphokines.

E. As a Drug Carrier

- conjugates of Con A with antitumor drugs such as daunomycin, chlorambucil and methotrexate have been tested *in vitro* with cultured tumor cell lines.
- Con A linked to the α chain of diphtheria toxin or ricin.

F. Clinical Uses

- blood typing.
- determination of microheterogeneity of α-fetoprotein in amniotic fluid, for the prenatal diagnosis of fetal neural tube defects and other malformations.
- assess the immunocompetence of patients through the use of mitogenic lectins.
- removal of mature T-cells from human bone marrow by soybean agglutinin, or by ricin linked to monoclonal anti-T cell antibodies.

PNA binds to both the α and β configurations of thi. disaccharide, which are the immunodeterminant groups of the T and asialo GM_1 antigen respectively[163,164]. PNA also binds to other similar or closely related carbohydrate structures[166]. The T-antigen is an important tumor-associated marker and is found in an exposed form in a wide number of human carcinomata, but is masked by sialic acid on corresponding benign or healthy tissues. Thus PNA may be valuable as a potential tumor imaging agent or as a carrier for chemotherapeutic or radioactive nuclides to the T-antigen expressing tumor. PNA binding has been demonstrated histochemically in a wide variety of T-antigen expressing adenocarcinomas including lung, gastrointestinal tract and breast tumors but not in the corresponding normal cells. *In vivo* studies with radioiodinated PNA in animal tumor models also showed significant tumor uptake at 48 to 72 hours post injection[104].

2.4.1 Isolation and Purification of PNA

The isolation of PNA from peanuts begins with a crude saline extract (10% w/v NaCl solution) which takes out most of the protein components[165] . In addition to proteins, other lipid components (up to 40% of their weight) are also extracted, so defatting of the extract is required with organic solvents such as ether or acetone[165]. The protein from the defatted saline extracts are then salted out with a neutral salt (saturated ammonium sulphate). After centrifugation, reconstitution of the insoluble fraction, dialysis and/or ultracentrifugation, only PNA and a few peanut proteins will be present[165,166]. Affinity chromatography is then used to separate PNA from these other peanut proteins. Various adsorbents are available for the affinity chromatography. These usually consist of an immobilized carbohydrate structure which binds PNA specifically and the PNA may then be desorbed with a sugar solution that will compete for the same binding site as PNA[165-167].

Another method which does not utilize carbohydrate specificity is by precipitation of extraneous protein from a defatted peanut extract with a dilute solution of Rivanol® (2-ethoxy-6,9-diaminoacridine lactate)[168]. This method is simple, rapid and the purified PNA

was found to be serologically as active as the PNA obtained from some of the affinity chromatographic process[168].

2.4.2 Macromolecular Properties

The molecular weight of affinity purified PNA has been determined by several investigators. Estimates of the aggregate molecular weight of the lectin have been reported as 98,000 daltons, 106,500 daltons and 110,000 daltons as determined by gel filtration, sedimentation velocity and by sedimentation equilibrium centrifugations[165,169,170]. The discrepancies found in the reported values have been attributed to several possible factors such as the variation in extracting methods resulting in slight aggregation, concentration of the PNA used in the study and/or isolectin variation[170].

PNA is a relatively acidic (pI=5.95), hydrophilic and compactly folded protein[165]. It is composed of four identical noncovalently linked subunits, with molecular weights of 24,500 to 28,000 depending on the technique employed[165,169]. The stability of the tetrameric structure is pH dependent and at pH 3.0 PNA reversibly dissociates to a species of about 48,000 daltons which is devoid of sugar binding capacity[165]. In the presence of detergents or denaturing conditions PNA dissociates to 4 identical subunits of 27,000 daltons in which the last five amino acid sequences from the amino terminal are identical[165,169].

Salunke *et al* [171] suggested that the tetrameric structure of PNA is a dimer of a dimer. The monomers in each dimer are related by a two-fold axis. The two dimers are in turn related to each other by another two-fold axis perpendicular to the axis of monomers in the dimer. The molecule thus has 222(D_2) symmetry[171].

Crystallization and preliminary x-ray crystallographic studies of the peanut lectin have been reported[162,172]. Three crystalline forms (monoclinic I and II and a triclinic form) were reported to contain approximately 50% water[172]. The secondary structure of peanut lectin is dominated by β-pleated sheets as determined by circular dichromic spectra measurements[173]. The hydrodynamic radius of the molecule has been estimated as 35.5±1.5 A[162]. Ultraviolet

spectroscopy reveals a small absorption peak at 290 nm and a double maxima at 277 and 283 nm[169]. The extinction coefficient is calculated to be 0.96 at 280 nm and the absorption coefficient (A_{280}) is 7.7 cm^{-1} [169]. Saccharide induced transition is obtained from the UV spectrum in the presence of inhibiting sugars. The binding of PNA to β-D-Gal(1→3)GalNAc occurs with an entropy (S) of -177±16 J/Mole. K and an enthalpy of -78±5 KJ/mole[174].

Peanut lectin contains one atom each of Ca^{+2} and Mg^{+2} per subunit and it does not contain any covalently bound carbohydrates[175]. Amino acid sequencing studies of PNA have been reported by several investigators. Lotan *et al* [166] found PNA to be rich in acidic and hydrophilic amino acids, with relatively little methionine, tryptophan, histidine and an absence of cysteine. Terao *et al* [165] reported different values with considerably less threonine, serine, tryptophan, agrinine, an absence of methionine, but with 16.6 moles of cysteine per mole of PNA. The amino acid sequence of the N-terminal has been worked out to forty residues.

Studies of PNA stability have shown that this lectin is quite stable under a wide variety of conditions. It is stable within the concentration range of 0.5 to 2.0 mg/ml in PBS[176]. Cryoinsolubility has been noticed with PNA solutions of high concentration (>2 mg/ml) at temperatures below 4°C [176]. This is most likely due to protein insolubility as polymerization or conformational changes are not observed. The presence of β-D-Gal(1→3)GalNAc results in preservation of solubility[176].

PNA is very stable both in solution and as a lyophilized powder. Crude saline extracts of PNA have shown no significant loss of agglutination ability when stored at 20°C for up to 6 months while reconstituted PNA (1 mg/ml in the presence of bovine serum albumin) from lyophilized powder is stable for up to 2 years stored at 4°C [81].

2.4.3 Isolectins

Affinity purified PNA is found to contain multiple molecular forms under polyacrylamide gel electrophoresis and isoelectric focusing[170,177]. These multiple molecular forms have very similar isoelectric points and are known as isolectins. All of the isolectins

found were reported to consist of subunits of similar molecular weight (28,000) and these isolectins agglutinate neuraminidase-treated red blood cells. Cross-reactivity to a murine anti-PNA antibody was similiar for each isolectin studied[170,177].

A large-scale screening of peanut and its wild relatives (4556 genotypes) revealed the presence of 6 to 8 separate isolectins. All but four of the wild species of *Arachis* seeds contained PNA[170]. PNA isolated from the variety "Shulamit" is mitogenic towards desialyzed lymphocytes, while PNA isolated from other genotypes has not been found to be mitogenic[178]. The slight difference in isolectin properties may account for the minor differences observed when PNA interacts with cellular glycoproteins.

2.4.4 Carbohydrate Binding Specificity of PNA

Extensive studies on the carbohydrate-binding properties of peanut agglutinin employing hapten-inhibition and spectroscopic techniques indicated that the lectin possesses an extended binding site that is specific for galactosyl end groups and recognizes the T-antigenic structure (Gal-β(1→3)GalNAc) [165,169,179-181]. Earlier studies employing hemagglutination inhibition also show this disaccharide to be a very effective inhibitor[161]. It is also suggested that the peanut lectin receptor is part of the base-labile oligosaccharide (Neu5Acα2,3Galβ1,3[Neu5Acα 2,6] GalNAcα1-O-Ser, Thr) which is responsible for the MN blood group activity[169]. Desialization by neuraminidase or by acidic hydrolysis unmasks the PNA receptor.

Many researchers have studied the inhibitory effect of carbohydrate moieties on PNA hemagglutination or glycoprotein precipitation. The results are summarized in table 9.

Using the information obtained from carbohydrate inhibitory studies illustrated in table 9, some generalizations can be made concerning the structural features of carbohydrates that are required for binding or inhibition of PNA agglutination:

1. A methoxy group on C-1 enhances binding to PNA, and the α anomer seems to have slightly better binding than the β anomer. The O-1 atom seems to be involved in the

Table 9

Inhibition of PNA Hemagglutination or Glycoprotein Precipitation by Carbohydrates.

Inhibitor	Relative Inhibitory Potency	
	Precipitation (a)	Hemagglutination (b)
Galactose	1.0	1.0
L-Fucose	0.60	<0.25
Galactosamine	2.22	1.43-4.25
N-Acetylgalactosamine	<0.10	
Methyl α-Galactoside	2.22	
Methyl β-Galactoside	1.50	1.25
p-Nitrophenyl α-Galactoside	2.22	
p-Nitrophenyl β-Galactoside		
Galβ(1→3)GalNAc (T-antigen)	54.5	50
Galβ(1→3)GAlNAcOH	2.20	
Galβ(1→3)GlcNAc	0.60	
Galα(1→3)Glc	2.22	1.66-2.0
Galβ(1→6)Glc	2.22	
Galα(1→6)Glc	0.60	0.80
MN glycoprotein		2.5
Asialo MN glycoprotein		5000
Ch-3 (MN glycoprotein fragment)		20.0
Asialo Ch-3		5000

Galactose activity is normalized to 1.0.

(a)Concentration required for 50% inhibition of precipitation of blood group glycoprotein (Pereira *et al*[111]).

(b)Concentration required for 50% inhibition of B-type N'RBC Agglutination (Lotan *et al*[66]).

glycoside bonding and the second residue is involved in the association with PNA. The $\beta(1\rightarrow4)$ configuration shows better binding to PNA than the $\alpha(1\rightarrow6)$ configuration , suggesting that the C-1 configuration is also important[169,181,182].

2. At the C-2 position, a group which is capable of forming hydrogen bonds, such as a hydroxyl or amino acid group, is required for binding[179,182].
3. A free hydroxyl on C-4 is necessary for binding, and the configuration of the C-4 terminal residue should be similiar to D-galactose[169].
4. The C-5 hydroxymethyl group is also important in binding to PNA[182].
5. An extracyclic chain on C-6 is necessary for binding and the orientation of the C-6 hydroxyl group is critical[182].
6. Terminal sugars in the pyranose form are more effective inhibitors than those in the furanose or 'open chair' form[169].

Jimbo and Matsumoti[183] observed that the tyrosine residues of PNA are being perturbed upon the binding of carbohydrate ligands. Lactose will protect some tryosine residues from modification by iodine, while free amino groups do not appear to be involved in carbohydrate binding.

PNA-glycoprotein, glycolipid or polysaccharide interactions are more complex than that seen with simple mono- or disaccharides. Other secondary interactions can also occur outside of the primary binding site. These mechanisms include multivalent binding, steric interaction and non-specific binding[183].

A two-step binding mechanism for PNA-ligand interaction has been proposed by Neurohr *et al* [184,185]. It involves a initial interaction followed by conformational rearrangement resulting in the final complex. Changes in the circular dichromic spectrum of PNA are produced by the binding of simple glycosides and complex glycopeptides, and more pronounced conformational changes are observed with binding to the simple sugars. This suggests that the extended binding site for glycoprotein may be predetermined and that simple sugars should have lower binding constants. This suggestion is consistent with the results

obtained from the inhibition studies.

2.4.5 Biological Binding of PNA

PNA interacts with the T-antigen and with desialated glycoproteins and glycolipids containing this antigenic determinant. This makes PNA a useful tool as a cell surface probe in a number of histological and immunological studies. The binding of PNA to a variety of tissues and cell populations has been reported[116]. The interaction of PNA with neoplasias is of particular interest and its binding to a number of T-antigen bearing tumors has been shown.

2.4.5.1 Red Blood Cells (Erythrocytes)

PNA is used routinely in blood bank procedures for the detection of the T-antigenic disaccharide β-DGal(1→3)DGalNAc α1-linked , which is the mucin type carbohydrate sequence normally found in a cryptic form on human erythrocyte membranes[117]. The distribution of PNA positive cells in human peripheral blood is quite low (about 5%), most of which appear to be monocytes[118]. However, neuraminidase treated erythrocytes, irrespective of their ABO blood group, will be agglutinated by PNA. The presence of the Thomsen Friendenreich cryptoantigen on the erythrocyte membrane has also been demonstrated by other serological and chemical methods.

Several investigators have suggested the presence of two distinct antigens on erythrocyte membranes when treated with neuraminidase, those with terminal D-galactose and those with terminal N-acetyl-D-galactosamine[166,119]. PNA only has affinity for the exposed antigen with the terminal D-galactose residue[166]. This may explain the difference in binding observed between young RBC and senescent RBC[82]. Aged RBC, and cells with extruded nuclei do not bind PNA to the same extent as normal cells. *In vivo* cleavage of exposed D-galactose by β-galactosidase after desialation may be responsible for the lower galactose content in older erythrocytes which in turn do not bind PNA as well[82].

2.4.5.2 Lymphocytes

The lymphoid system is a complex organization of numerous cell populations, such as the T and B lymphocytes which differ in their immunological functions. However, morphologically they are quite similar and various cells are often present in the same lymphoid tissue[190]. Researchers have identified different lymphocyte membrane antigens which are able to distinguish between the cell populations. Lectins, including PNA, have been used to identify these lymphocyte cell surface antigens and are used as a tool to separate the various lymphocyte subpopulations[191,192].

In humans, PNA will bind to peripheral blood lymphocytes only after neuraminidase treatment, and the same is observed with the peripheral blood lymphocytes of several other species, including rats, mice and guinea pigs[191]. PNA has also been shown to stimulate mitogenic activity in unmasked human and rat lymphocytes, but not in untreated cells[178]. In lymphoid cells, binding of PNA to immature thymocytes and some neoplastic lymphocytes is observed[191]. In mice, PNA receptors have been demonstrated on the membrane of thymus T lymphocytes of cortical origin. PNA also binds to suppressor T-cells and thus has been used for the differentiation and separation of suppressor-T cells from lymphocyte population[191].

There are a small number of cells in the bone-marrow, spleen and lymph nodes that will bind PNA[193]. The PNA-binding bone marrow cells in mice have been shown to be immature T-lymphocytes[191]. Human umbilical cord lymphocytes are also shown to be PNA positive. Thus, PNA may be useful in studying the ontogenesis of T-cells and the hematopoetic stem-cell maturity. PNA may also be a marker of immature B-cells since germinal centres in mice and human are also known to bind PNA[194].

Several lymphoblastoid lines of T or B cells also express PNA binding properties. Binding of PNA is reported in Burkitts' lymphoma and several T-cell lines of leukemia, including blast cells of acute lymphoblastic leukemia, stem cell leukemia and myeloid leukemia[195]. Lymphocytes of most chronic lymphatic leukemias do not bind PNA[193],

while peripheral lymphocytes of children with acute lymphoblastic leukemia were often positive[196]. PNA binding may be an indicator of poor prognosis in these cases but some reseachers have shown contradicting results[197].

PNA has also been shown to bind to platelets only after treatment with neuraminidase, indicating that platelets contain cryptic PNA receptors[198].

2.4.5.3 Binding to other Tissues

PNA binding sites are also found in human fetal colon[199]. PNA binds to the glycocalyx of columnar cells but not to fetal goblet cells. Treatment with neuraminidase renders the goblet cells PNA positive. The PNA binding pattern of fetal colon show certain resemblances to human colonic adenocarcinomas[199]. PNA will also bind to the actual goblet theca in normal adult human colon. This represents the detection of nascent oligosaccharides prior to the addition of terminal sialic acid[199].

PNA receptors are also present in the retinal extract of human and several mammalian species[200,201]. PNA binds consistently to domains of the interphotoreceptor matrix associated with the cone, but not with the rod. It suggests the presence of galactosyl and/or galactosaminyl residues in the mammalian cones, but not in the rods[200].

The alveolar lining of newborns with hyaline membrane disease has been shown to bind PNA, while no reaction with PNA was observed in control lungs[202]. Neuraminidase treatment did not alter the pattern of PNA binding. This may indicate the absence of terminal neuraminic acid on the alveolar lining and suggests arrested maturation of the alveolar lining layer in this neonatal pathological condition[202].

2.4.6 PNA Binding in the Kidney

The *in vitro* binding of PNA in kidney sections of many animals and human has been reported[203-206]. There are considerable species to species variation in the binding pattern in various components of the kidney. In the mouse, binding of PNA is seen in the glomerulus, proximal tubule, loop of Henle, the distal tubule and the collecting ducts[203,204]. The binding

pattern in the rat is similiar to that seen in mice except that binding to the glomerulus is not observed. In other species, PNA binding in the kidney is more selective. In rabbit, binding is only observed in the distal tubule and the collecting ducts. In guinea pig, intense binding is seen in the S_1 segment of the proximal tubule, while in dogs, the distal tubule is the only component of the kidney that is stained positive[203,204].

The pattern of PNA binding in human kidneys has also been studied extensively[205]. Binding has been reported in the Bowman's capsule of the glomerulus, the luminal membrane of the ascending loop of Henle, the distal tubule, and to the collecting ducts[205,206]. The proximal tubules did not bind PNA even after neuraminidase treatment[205]. Children with haemolytic-uremic syndrome have strong glomerular binding of PNA, possibly due to the action of neuraminidase released from pneumococcal infections on the cryptic T antigen in the glomerulus[207].

Intense binding of PNA has also been reported in cases of hypernephroma[208]. Both cytoplasmic and membrane bound PNA receptors have been identified. If the hypernephromas are derived from epithelial cells of the proximal convoluted tubule, their binding may be explained by the malignant transformations resulting in the synthesis of altered membrane glycoconjugates which contain PNA binding sites. However, binding can also occur if hypernephromas are derived from other portions of the kidney which bind PNA[208].

The renal pharmcokinetics of intravenously injected radioiodinated PNA has been examined by Boniface *et al*[209] in dogs, rabbits and humans. In humans, the time to peak and percent injected dose-at-peak in kidneys has been calculated to be 90 minutes and 20.2±4.6% respectively[209]. In humans, PNA appears to be excreted largely through the renal tubules. The renal excretion mechanisms of PNA are still not very clear at the present, although filtration through the glomerulus is not likely, due to PNA's high molecular weight (about 107,000 daltons) and a pI of 5.8. Boniface *et al*[209] suggested a receptor-mediated tubular endocytosis/excoytosis mechanism which may be responsible for the renal elimination of PNA. The renal excretion characteristics of PNA may make it a useful tool for the *in vivo*

assessment of renal-tubular function in certain clinical situations such as monitoring of renal transplantation , and the effects of renal exposure to nephrotoxic drugs and/or radiation.

2.4.7 PNA Binding to Tumors

The expression of the T-antigen in a non-cryptic form has been demonstrated on a number of animal and human tumors. Since PNA detects the T-antigenic structure, it has been used extensively in a variety of histochemical and immunological studies of these T-antigen expressing tumors. The binding of PNA to various tumor tissue sections and to *in vitro* tumor cell cultures has been widely reported[210-235].

2.4.7.1 Binding to Mammary Carcinoma

The presence of non-cryptic, immunoreactive T-antigen on human breast carcinoma but not on corresponding healthy or benign breast tissues as determined by serological methods has been well documented[108,210-221]. Some researchers have utilized the humoral and cellular response to the T-antigen to differentiate carcinoma from non-carcinoma lesions.

The binding of PNA to the breast carcinoma has been studied extensively and some of the observations are summarized in table 10. Histochemical studies of T-antigenic structures with PNA reveals that free T-antigen is also expressed on some portions of the normal breast epithelium as opposed to the serological finding[108,210,219]. The luminal duct and lobular membranes are stained by PNA, and the intraluminal secretions in benign conditions occasionally bind PNA. The intensity of PNA staining appears to be dependent on the secretory state of the epithelium[215-217,220] .

The main difference between the binding pattern of PNA in benign and malignant cells is the transition from luminal to cytoplasmic staining. Several researchers have tried to correlate the PNA binding pattern with the histological grade of breast cancer but no significant relation can be established[210,213,214,219].

Table 10

Histological Evidence of PNA Binding in Mammary Carcinoma

Studies	Observations	References
Klein *et al*, 1979.	- free PNA receptors are observed on normal, hyperplastic mammary tissue as well as in carcinomas of the breast. - the binding intensity seems to reflect the secretory condition of the epithelium. - differentiated carcinomas bind PNA stronger than undifferentiated carcinomas of the breast. - the presence of free T-antigen on breast epithelium may be useful for diagnosis in histopathology of breast cancer.	108
Newman *et al*, 1979.	- a positive correlation is seen between PNA binding and the degree of differentiation in the breast epithelium. - different PNA binding pattern is observed between differentiated carcinomas or normal epithelium (cell periphery binding) and undifferentiated carcinoma (cytoplasmic binding). - PNA may be used as a marker for breast epithelical cell differentiation.	210
Howard *et al*, 1981.	- 19/22 benign breast lesions show PNA binding along the luminal membrane and occassionally on intraluminal secretion. - 17/22 breast carcinoma showed diffuse cytoplasmic PNA binding. - the immune response to T-antigen may be dependent on cellular localization of the antigen.	211,212
Hageman *et al*, 1983.	- PNA binding is also observed in the stroma probably due to binding to glycoprotein containing terminal non-reducing galactose. - no correlation is found between the presence of PNA receptors and the histological type of the tumor. - PNA positive lymphocytes are found in strongly stained stroma in cases of lymphocyte infiltrations.	213,214

Continued...

Studies	Observations	References
Klein and Vierbuchen, 1983.	- in rat mammary carcinoma, estrogens induced the biosynthesis and secretion of large amounts of PNA binding sites, and this effect can be inhibited by ovariectomy or by tamoxifen which will decrease the availiablity of the steroid receptors. - PNA may be able to distinguish between hormone dependent and hormone independent tumors. - tumors with a high secretory activity responded in about 80% to endocrine treatment while non-secretory breast tumor failed to respond to endocrine therapy.	215, 216, 217
Seitz *et al*, 1984.	- ~ 75% of confirmed metastases of mammary carcinomas showed positive cytoplasmic PNA binding and this may be useful for detection of single metastastic adenocarcinoma cells of the human breast.	218
Bocker *et al*, 1984.	- PNA binding pattern in 120 cases of invasive mammary carcinoms and 14 cases of normal or benign breast epithelium are studied. - statistically significant correlation are found between globular-vacuolar PNA-reaction and tumor type, and between PNA-histopositivity and estrogen/progesterone positive cases. - no correlation was found between PNA histochemistry, histological grading and pathological staging.	219
Walker *et al*, 1985.	- there seems to be a correlation between oestrogen receptor status and the reactivity of carcinomas to PNA.	220
Helle and Krohn, 1986.	- PNA receptor is a carbohydrate component of a milk protein in breast carcinoma. - monoclonal antibodies against human milk fat globule membrane antigens and PNA may detect overlapping antigenic epitopes in mammary carcinoma.	221

The binding of PNA to the mammary tissues is affected by the presence of steroid receptors and correlates with the responsiveness of the tumor to endocrine treatment[217,219].

2.4.7.2 Binding to Carcinomas of the Gastrointestinal Tract

Springer *et al* [14] reported that 42 % of patients with gastrointestinal carcinomas in his study had severely depressed levels of anti-T antibodies. This suggests the presence of non-cryptic T-antigenic structures in the gastrointestinal carcinomas. To demonstrate the presence of the T-antigen, the staining pattern of PNA with normal and malignant colorectal tissues were studied and some of the observations are summarized in table 11 .

The binding of PNA to normal gastrointestinal mucosal surfaces if present, is usually confined to the golgi region and in some cytoplasmic granules[223,225,227]. In the gastric mucosa, binding is observed within the isthmus, mucus neck, parietal, chief cells and the antral cardiac glands, while in the intestinal mucosa, binding is seen in the brunner glands, the columnar epithelial cells and the globlet cells of the small intestine[224]. The body of the stomach binds PNA only after neuraminidase treatment. Free or cryptic T-antigen is not observed on the rest of the normal colon, ileum and stomach[224].

An increase in PNA binding in the mucous, golgi body region and perinuclear region is observed in hyperplastic alterations of the gastric mucosa[225]. In large bowel carcinoma, the glycocalyx and cytoplasm of apical regions are shown to express free T-antigen[223]. It has been suggested that the increase in T-antigen expression is due to the decrease in glycotransferase levels in malignant cells resulting in incomplete biosynthesis of MN blood group glycoproteins[225,227].

2.4.7.3 Binding to Urinary Tract Tumors

The binding of PNA to urinary epithelium in histochemical studies is summarized in table 12. Normal as well as neoplastic urothelium posses "cryptic" PNA binding sites which are exposed by the action of neuraminidase[229,230]. Spontaneous binding to PNA is

Table 11

Histochemical studies of PNA binding sites on gastrointestinal carcinomas

Studies	Observations	References
Boland *et al*, 1982.	- PNA labels goblet cell mucins in colon cancer specimens but not with normal colon. - transitional mucosa also contained goblet cell mucins that are labelled by PNA. - the presence of PNA binding carbohydrate structures in the mucin of transitional mucosa suggests that this tissue may be in the process of early malignant transformation.	222
Cooper, 1982.	- PNA binding sites are observed in the region of the glycocalyx and in the apical portion of the cell in rectosigmoid carcinomas. - the more poorly differentiated carcinomas less frequently express PNA binding sites. - reduced level of tumoral glycosyltransferases may explain the increases in non-cryptic T-antigen in the carcinomas.	223
Fischer *et al*, 1983.	- PNA binding in gastric epithelial cells seems to be associated with the early phase of glycoprotein synthesis. - there is an intense PNA binding to embryonic, hyperplastic and carcinomatous gastric mucosa.	224
Cooper and Reuter, 1983.	- approximately 80 % of tubular adenomas and villous adenomas studied express T-antigen. - two patterns of PNA binding are observed. - the supranuclear pattern is similiar to the pattern observed in non-neoplastic colonic epithelium with complete MN blood group glycoprotein. - the apical cytoplasm and/or glycocalyx pattern corresponds to fetal or carcinomatous epithelia which are deficient in glycosyltransferases and form incomplete glycoproteins.	225,227

Continued...

Studies	Observations	References
Picard and Feizi, 1983.	- PNA reacts with non-neoplastic gastric glycoproteins of "non-secretors" but not with those of "secretors". - the expression of PNA binding sites in gastric glycoproteins is dependent on secretor status. - colorectal tissue extracts do not react with PNA. - gastric tumor extracts reacts with PNA regardless of secretor status.	226
Orntoft *et al*, 1985.	- T-antigen are commonly expressed by carcinoma cells of the colon and rectum but not by the normal mucosa. - the staining pattern in tumors are heterogenous. In some tumors all malignant cells are stained, while in others, stained cells alternated with unstained cells. - T-antigen may occur early in the malignant transformation and could be of potential value in the identification of patient at risk.	113
J.C. Macartney, 1986.	- PNA binding to surface mucous cells in normal gastric mucosa is seen in non-secretors but not with secretors regardless of ABO group. - extensive masking of lectin binding sites by sialic acid is seen in gastric cancers.	228
Yuan *et al*, 1986.	- PNA and monoclonal anti-T antibody binding to normal, premalignant and malignant human colonic tissue is compared. - PNA has the best sensitivity (91%) in cancer tissues but low specificity (68%) in normal mucosa. - PNA binding sites is observed in some non-neoplastic disease control.	112

Table 12

Histochemical studies of PNA binding sites on urinary carcinomas

Studies	Observations	References
Alroy *et al*, 1982.	- PNA binds to urothelium in the human urinary bladder only after neuraminidase treatment.	229
Coon *et al*, 1982.	- non-cryptic T-antigenic structure is seen in neoplastic but not on normal urothelium. - some cases of transitional cell carcinoma are stained by PNA, but some possess cryptic T-antigen or even the absence of T-antigen. - the PNA staining pattern is highly variable. - correlation between T-antigen status and histologic grade is highly significant. - low grade tumors are usually cryptic T-antigen positive, and with a lower incidence of invasion and recurrance. - high grade tumors are usually T-antigen positive or cryptic T-antigen negative.	230
Summers *et al*, 1983.	- a number of factors, including T-antigen status are studied as prognostic indicator for bladder transitional cell carcinoma. - patients with cryptic T-antigen positive carcinoma generally did not suffer invasive recurrences. - combination of markers are more effective than any single marker system.	110
Lehman *et al*, 1984.	- T-antigen expression is most likely due to incomplete synthesis of MN glood group glycoproteins. - there is also evidence of cytostructural relocalization of the T-antigen.	227
Vafier *et al*, 1984.	- T-antigen expression is not completely useful prognostically, it does not correlate with grade, stage or clinical course of transitional cell carcinoma. - ABO(H) antigen expression may be a better predictor of tumor behavior.	111

Continued...

Studies	Observations	References
Kagawa *et al*, 1985.	- T-antigen status did not correlate with grade, stage of tumor and survival of patients in upper urinary tract tumor.	96
Limas and Lange, 1986.	- non-crypt. T-antigen is absent in normal bladder epithelium, but present in 10% of the non-invasive and 65% of invasive transitional cell carcinoma. - PNA staining is seen in cytoplasm, cell surface and mucin. - expression of T-antigen may occur later when the tumor has advanced to invasive stages. - the presence of free T-antigen will increase the risk for metastatic involvment of regional lymph nodes.	109

not observed in the normal bladder epithelium, but is observed in a small number of non-invasive and a large proportion of the invasive transitional cell carcinomas[229]. The staining is observed in the cytoplasm, cell surface and in mucin[109].

The expression of the free T-antigen may reflect the neoplastic transformation in the bladder epithelium. Some researchers have suggested that the T-antigenic status may be used as an indictor of prognosis in small cell transitional carcinoma[230]. However, other researchers found that ABH blood group antigens correlate with tumor grade, stage and survival and may be a better prognostic indicator than the T-antigen[96, 111].

2.4.7.4 Binding to Other Neoplasias

The binding of PNA has been studied with a number of different tumor types. In adenocarcinoma of the prostate, the histochemical binding of PNA to benign hyperplasic and the malignant lesions has been reported[231]. In benign lesions, most of the studies did not reveal any free T-antigenic binding sites in the grandular epithelia but the presence of cryptic T-antigen was demonstrated by the action of neuraminidase. In adenocarcinomas, the tumors may be T-antigen positive, cryptic T-antigen positive or may not posses any T-antigen binding at all. Using PNA as an *in vitro* histochemical probe, a correlation was observed between the status of T-antigen expression and the tumor grade as well as metastases in patients with prostate carcinoma[231].

Surface antigens on embryonal carcinoma cell are observed to undergo significant changes during *in vitro* differentiation[232]. PNA binding to murine embryonal carcinoma cells detected the changes in localization and distribution of the PNA reactive antigens during the developmental stages. PNA will bind to murine embryonal carcinoma cells but the binding is lost following *in vitro* differentiation[232].

PNA did not bind to normal cervical squamous epithelium but strong focal membranous and cytoplasmic staining were observed in atypical cells and carcinoma *in situ* [233]. Invasive squamous cell carcinoma showed variable cytoplasmic and membranous interaction with PNA. The atypical cells in adenocarcinoma of cervical squamous

epithelium *in situ* were negative to PNA binding probably due to the lost or altered secretory activity due to the malignant transformation[233].

Histocytosis X is characterized by a proliferation of Langerhans' cells thought to be due to a disorder in the immune system[234]. The PNA binding pattern in Histocytosis-X can be used as a marker to differentiate it from malignant histocytosis and macrophage histocytosis[234]. PNA showed unique paranuclear and cell surface staining of histocytosis-X cells while benign macrophage histocytosis cells showed a diffuse cytoplasmic staining pattern. The malignant cells of malignant histocytosis were not stained at all by PNA. Similiar findings were observed with eosinophilic granuloma of bone[235]. A unique membrane and cytoplasmic (Golgi) PNA staining pattern was shown on cells of Langerhan's origin, and this pattern may be used as a simple and quick tool for routine diagnostic evaluation of suspected eosinophilic granuloma[235].

2.4.8 *In Vivo* Biodistribution Studies with PNA

Zabel *et al* [101] were the first to report the biodistribution of radioiodinated PNA in an animal tumor model. CBA/CAJ mice bearing the T-antigenic expressing RI lymphoma were used as the animal tumor model. Following intravenous injection of I-125 labelled peanut lectin, a significant uptake of the radiolabelled lectin was seen in the kidney and tumor (tumor to blood ratio 7.5 : 1 at 72 hours post injection). This was in agreement with the *in vitro* binding studies which showed a specific binding of PNA to this cell line. However, there was also rapid *in vivo* deiodination resulting in the uptake of free radioiodide in thyroid, salivary glands and stomach[101].

Further biodistribution studies in other murine tumor models were carried out by Shysh and Eu *et al* [236,237]. They observed a even more pronounced uptake of radioiodinated PNA in strain AJ mice bearing the subcutaneously implanted TA_3/Ha tumor. Tumor to blood ratios of 70-85 : 1 at 72 hours post injection were observed. The high tumor to blood ratio was explained by the rapid blood clearance and renal excretion of the lectin. Clear gamma

camera scintigraphic delineation of the tumor site was obtained without any background subtraction technique. The lung and liver metastasic variant of the TA_3/Ha cell were selected and implanted into strain AJ mice resulting in definite lung or liver metastases. Clear delination of the specific metastases were also obtained by gamma-ray scintigraphy. Rapid *in vivo* deiodination are also observed with uptake noted in the thyroid and stomach.

Acute toxicity studies in mice with I.V. injections between 50 to 3600 ug of PNA did not reveal any abnormalities using weight gain as a control. Histochemical examination at the light microscopal level of mouse organs after the injection also confirmed the findings[104].

The *in vivo* uptake of radioiodinated PNA in other tumor model were studied by Yokoyama *et al* [238]. Superior delineation of Lewis lung carcinoma and Ehrlich ascities tumor were obtained by PNA compared to Ga-67 citrate, and similiar uptake was observed in Yoshida sarcoma and in B-16 melanolic melanoma. They attributed the superior performance of PNA over Ga-67 citrate due to the lack of PNA accumulation in abscesses.

Holt *et al* [239] studied the biodistribution of I.V. administered radioiodinated PNA in cancer patients. Eight patients with metastatic cancer of the colon, lung or breast were injected with 37-93 MBq (17-88 ug) of I-131 PNA. Rapid clearance of the radiopharmaceutical was observed with $82.5 \pm 5.3\%$ of the activity excreted in the urine within the first 24 hours post injection. The renally excreted I-131 PNA appeared to be intact and immunoreactive as determined by binding to asialo GM_1 synsorb and gel column chromatography. Gamma scintigraphic studies revealed known metastatic sites in only two patients, and in a further 2 patients, an adjacent malignant pleural effusion was visualized. In other cases, the known metastatic sites did not show up in the scans. Although the results in tumor scintigraphy were quite disappointing, the researchers suggested that PNA may have a role in the assessment of renal tubular function due to its unique renal excretion mechanism.

Further human studies were conducted by Abdi *et al* [240] in 17 patients with proven metastatic cancer (breast, colorectal, small cell lung carcinoma, renal cell carcinoma, or adenocarcinoma of the lung). Renal accumulation of I-131 PNA was apparent shortly after

the I.V. injection with a mean percentage of the injected dose at peak of 20.5±0.7% in three patients who exhibited normal renal uptake of I-131 PNA. Interestingly, only 1 patient exhibited uptake of I-131 PNA at the site of a known metastatsis and this patient had a markedly reduced renal lectin uptake. The rest of the patients did not show sufficient uptake of the radiotracer at the known metastatic sites to provide a useful scan, nor did this procedure reveal other lesions not detected by other conventional diagnostic methods.

In all the patients studied, no significant immediate or short-term side effects (hypersensitivity or anaphylactic reaction) due to the intravenously administered of radioiodinated peanut lectin had been noted[240].

2.5 Radiolabelling of Protein

2.5.1 Radioiodination

Radioiodination is one of the most widely used methods for radiolabelling of protein. The techniques used to incorporate the iodine atom into proteins have been extensively reviewed[241,242]. The procedure usually involves the addition of iodine atoms mainly into tyrosine and occasionally into histidine, tryptophan or sulphydryl groups of the protein. Several isotopes of iodine are available thus offering a number of choices in terms of half-life and radiation characteristics for imaging or therapeutic purposes. Some of the common radioistopes of iodine and their characteristics are listed on table 13.

Radioiodine must be in its active or oxidized form for labelling. An oxidizing agent is usually used to convert the radioiodide into radioiodine which in turn reacts with water to form the the hydrated iodinated ion which is the reactive species[241,242]. Many methods have been used for the oxidation of the radioiodide, each with its advantages and disadvantages. Table 14 is a brief summary of the procedures used in radioiodination. In order to mininize the damage to the protein being labelled, the oxidizing conditions must be mild. Recently, 1,3,4,6-Tetrachloro -3α,6α,diphenylglycouril or iodogen® has gained wide acceptance as a mild

Table 13. Characteristics of Commonly Used Radioisotopes of Iodine

Radioisotope	Half-Life	Decay Mode	Major γ-Ray Energy in KeV (%)
I-123	13.2 h	E.C.†	159 (82.8%)
			Te x-rays :
			27 (114%)
			31 (26.0%)
I-125	60.1 d	E.C.†	35 (6.7%)
			Te x-rays :
			27 (114%)
			31 (26.0%)
I-131	8.0 d	β^-	80 (2.6%)
			284 (6.1%)
			364 (81.2%)
			637 (7.3%)

† E.C. - electron capture.

Table 14. Techniques Commonly Used for Radioiodination of Proteins[241,243]

1. DIRECT LABELLING METHODS.

A. Iodine Monochloride

- ICl is mixed with radioiodide and reacted with the protein for about one minute.
- the reaction is terminated by the addition of excess sodium metabisulfite.
- lower specific activity is obtained since stable iodide is also incorporated into the protein.
- protein is being exposed to potentially harmful radioiodide and reducing agents.

B. Chloramine-T

- widely used for radioidination of a variety of proteins.
- the reaction is terminated by the addition of sodium metabisulfite.
- optimal reaction is obtained at pH 7.
- high degree of iodine incorporation and high specific activities can be achieved.
- but protein damage and aggregation may also occur.

C. Chlorine Oxidation.

- chlorine gas (produced by chloramine-T and NaCl) is diffused into the reaction chamber containing the protein and radioidide.
- the reaction is terminated by the addition of sodium metabisulfite.
- the exposure of protein to harmful reagents is reduced.

D. Electrolytic Iodination.

- an electrolytic cell with a platinum crucible as the anode and a platimum cathode is used to generate the radioiodine.
- the procedure is mild and gentle to the protein.
- but the reaction is complex and specialized equipment is required.
- prolonged exposure to radioiodide and the temperature of electrolysis may damage the protein.

E. Enzymatic Iodination.

- lactoperoxidase is used to oxidize radioiodide in the presence of small amounts of hydrogen peroxide.
- reaction is quenched by cysteine or by dilution.
- pH of 5 to 6 is optimal for the reaction.
- denaturation of the protein is found to be minimal.

F. Iodogen®

- iodogen is coated onto the reaction vessel and the protein and radioiodide are added to the vessel.
- reaction is terminated by removal of the contents and no reduction step is required.
- the reaction is gentle and easy to perform.

2. CONJUGATION LABELLING METHOD

- 2 step method.
- labelling yield is usually lower than direct labelling methods.
- can target the labelling to specific amino groups.
- decrease the protein exposure to harmful reagents.

A. N-Succimidyl Hydroxyphenyl Propionate (N-SHPP)

- N-SHPP is first radioidinated by the chloramine-T method.
- reacts with primary amino groups of protein.
- the technique is time consuming and labelling yield yield of 10 to 30% has been reported.

B. Radioiodosulfanilic Acid Conjugation

- reacts with various amino acid residues in whole protein molecule.
- labelling yield of 80% has been reported.

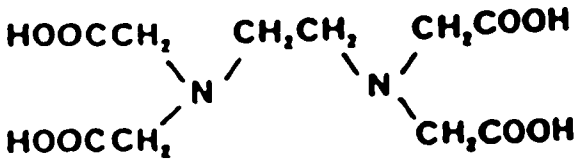
oxidizing agent for the radioiodination of a variety of proteins[244]. The reaction is simple, rapid and does not require a reduction step as the iodogen is coated onto the reaction vessel. Denaturation of the protein by this labelling method has been found to be minimal.

Certain problems have been encountered with the use of radioiodinated protein. *In vivo* deiodination is known to occur releasing free radioiodide from the protein. The free radioiodide will then localize in the thyroid, stomach and intestine, salivary glands and choroid plexus. This greatly limits the utility of the radiolabelled protein as the scintigraphic scan will degrade with time following administration and background subtraction techniques may have to be employed to visualize the region of interest. Another concern about the use of radioiodide is the damage to the protein being labelled. The protein is subjected to oxidizing and reducing conditions and may be in contact with the radioiodide for a considerable amount of time in some cases. Sufficient or critical damage to the protein will result in the loss of biological or immunological activities and the subsequent rapid clearance of the protein from the circulation, usually by the liver, after intravenous administration[241,242]. Thus it is important to compromise between specific activity and the biological or immunological activity of the protein to obtain a useful product.

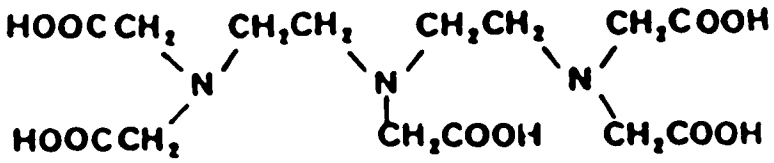
2.5.2 Bifunctional Chelating Agents

The first report of attachment of metal binding groups to proteins is that of Gelewitz *et al* [245] who coupled azo-phenanthroline and azo-oxine to albumin in 1953. Since then, many new bifunctional chelating agents have been synthesized, the major area of interest being on the derivatives of diethylenetriaminepentaacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA), whose structures are shown in figure 3. With the recent advance in monoclonal antibody technology, there has been a considerable amount of interest in the attachment of these bifunctional chelating agents to the antibody, thus providing a metal binding site which can then be subsequently labelled with a variety of radiometallic ions.

Figure 3. The Chemical Structure of Two Commonly Used Bifunctional Chelating Agents (DTPA and EDTA)



Ethylenediaminetetraacetic Acid (EDTA)



Diethylenetriaminepentaacetic Acid (DTPA)

The radiometals most commonly used for the chelation reaction are the isotopes of indium, gallium and technetium. In-111 has a 2.8 days half-life and major γ emissions of 172 and 247 KeV which are suited for imaging with the gamma-camera. In-111 has been widely used in labelling chelate coupled monoclonal antibodies. In-113m is a short-lived radioisotope (1.7 hours) available from a generator source. Its short half-live is not very suitable for labelling antibodies but it has been used as the EDTA and DTPA chelates for brain imaging and renal function studies [246]. Gallium-67, with its 3.3 day half-life and suitable γ emissions, has been used for labelling of monoclonal antibodies. Gallium-68 is a short-lived, generator produced, positron emitting radioisotopes that has been used in tomographic studies of liver brain. Tc-99m is a generator produced radioisotope with a 6 hour half-life and excellent radiation characteristics for scintigraphy. Its short half-life is not very suitable for imaging with antibodies since the optimal imaging times ranges from 3 to 5 days post injection. However, the short lived radionuclides may be useful for labelling monoclonal antibody fragments which are cleared more rapidly from the body thus allowing imaging at about 24 hours post injection [247,248].

Other radioisotopes, such as Re-186 or Y-90 can also be attached *via* the ligand chelation procedure thus providing compounds for therapeutic applications.

In addition to allowing for a wider choice of radioisotopes, the bifunctional chelating agents can also circumvent the problem of *in vivo* dehalogenation which severely limited the utility of radioiodinated compounds. Also, a convenient kit preparation of the chelated protein is possible, and the labelling can be performed in a single step by simply adding the radioisotope of choice.

2.5.3 Development of the Bifunctional Chelating Agents

Pioneer studies by Goodwin *et al* [249] and Sundberg *et al* [250,251] with azo-phenyl EDTA showed that the coupled proteins were stable enough for *in vivo* use. Although other chelating agents such as transferrin [252], D-penicillamine [253] and deferoxamine [254] have been successfully

coupled to proteins *via* glutaraldehyde or carbodiimide, the most commonly used bifunctional chelating agents remain to be the derivatives of DTPA and EDTA.

Goodwin *et al* [249] derivatized EDTA by the addition of a nitrophenyl group and subsequent conversion into a diazonium salt. The diazo group reacts with terminal amino groups on the protein forming a stable covalent bond. The In-111 labelled diazo-phenyl-EDTA protein was stable *in vitro*, however, its use was limited by the complicated synthetic scheme and these complexes showed a faster biological clearance than equivalent radioiodinated proteins [255]. Other modifications of the nitrophenyl group such as acylation and alkylation have been studied and protein coupled through the alkylating group exhibited plasma clearance rates identical to those seen with the equivalent radioiodinated proteins[256,257].

In 1979, Yeh *et al* [258] devised a general method for converting α-amino acids to bifunctional chelating agents. This method provides a much simpler route for the production of nitro-phenyl and nitro-benzyl EDTA and permits the synthesis of a wide range of reactive functional group from these two parent structures. Meares *et al* [259,260] and Goodwin *et al* [261] utilized the *p*-bromoacetamide and the isothiocyanate derivaties of EDTA for coupling of mouse monoclonal antibodies. Both *in vitro* and *in vivo* studies with the In-111 labelled antibody showed that the protein retained its immunoreactivity as well as a minimally radioiodinated antibody. The isothiocyanate derivative is specific for amino groups while the haloacetamide derivative reacts readily with sulfhydryl groups on proteins, but will also react with an amino group which is thought to be the dominant site of reaction [260].

Recent research with EDTA derivatives involved the synthesis of chelates that have a strong enough stability constant to prevent transchelation in the serum which is a major source of instability for the chelated proteins [262]. The derivatized EDTA can be attached to the protein by reaction with carbodiimide. These newly synthesized EDTA chelates included propionic acid substitued ethylenediamine N,N'-di-[(*o*-hydroxyphenyl)acetic acid] and N,N'-dipyridoxylethylenediamine N,N'-diacetic acid (PLED) [263]. Another approach by Haner

et al [263] took advantage of the presence of a dangling carboxylate arm in the $[Co(EDTA)]^{2-}$ complex anion and conjugated it to *p*-aminobenzamide *via* carbodiimide. The cobalt ion can then be removed by reduction with Fe^{2+} and ascorbate and alternative metal ions inserted in its place [263].

Derivatives of DTPA have been used routinely for labelling monoclonal antibodies with In-111. The mixed carboxycarbonic anhydride of DTPA was first introduced by Krejcarek and Tucker [256] and adapted by several other researchers for labelling monoclonal antibodies. The mixed carboxycarbonic anhydride of DTPA is quite unstable and must be prepared freshly very time before conjugation to the protein [257,264]. The most popular bifunctional chelating agent used for coupling protein so far has been the bicyclic anhydride of DTPA. The major advantage for the bicyclic anhydride over the mixed anhydride of DTPA is that it is quite stable when stored desicated [265,266]. The bicyclic anhydride of DTPA was first described by Eckelman *et al* [265], but was modified and studied extensively by Hnatowich *et al* [266-268] to label a number of monoclonal antibodies. The conjugation to the protein is through available free amino groups. The amino group of lysine is usually the most readily available and forms a stable amide bond. The labelling yield, immunoreactivity and *in vivo* biodistribution of an anti-melanoma monoclonal antibody using the two derivatives of DTPA were similar [269]. The cyclic anhydride of DTPA possess two protein reactive groups, and problems arising from the cross-linking of protein molecules may occur. On the other hand, the DTPA mixed anhydride, under strictly controlled conditions, can be synthesized with only one protein reactive group, thus minimizing the problem of formation of high molecular weight forms of the protein [270].

The optimal reaction conditions for the conjugation of DTPA bicyclic anhydride to antibodies was studied extensively by Hnatowich *et al* [266-268]. Certain parameters, including the pH of the buffer used, the starting antibody concentration and the DTPA to antibody ratio were all shown to determine the number of chelates that can be attached to the antibody molecule. Hnatowich *et al* concluded that a high antibody concentration (>20 mg/ml) and a

pH of approximately 8.5 is required for high coupling yield.

Other methods for incorporation of DTPA into the protein molecule included directly linking DTPA to the protein with carbodiimide and the use of the N-hydroxysuccinimide ester of DTPA [271]. Najafi and Hutchison [272] studied the use of the DTPA N-hydroxysuccinimide pentaester as an alternative for the bicyclic anhydride for coupling protein with DTPA. *In vitro* studies showed more high molecular weight forms produced and decreased serum stability compared to the bicyclic anhydride labelled protein. This is expected due to the number of reactive groups available for cross-linking of the protein. This problem is mininized by using diactivated ester which gives better *in vitro* protein coupling and better *in vivo* stability [272].

Many other new chelating agents are being investigated at the present time, Buckley *et al* [273] used the chelating agent triethylenetetraminehexaacetic acid and coupled it to a anti-HCG monoclonal antibody by dicyclohexylcarbodiimide. Several new multidentate ligands designed for chelation with indium and gallium were prepared by Mattias *et al* [274]. These structures of these chelates are shown in figure 4. The chelates are lipophilic in nature and imaging and biodistribution studies in animals showed that they are cleared rapidly from the circulation and through the hepatobiliary system.

In addition to the development of new chelating agents for indium or gallium, there is also some interest in the labelling of monoclonal antibodies with Tc-99m through metal chelation. Tc-99m has excellent emission characteristics for use in imaging studies. Its 6 hour half life is not very suited to tumor imaging with intact monoclonal antibodies since the optimal imaging time is usually 2 to 3 days post injection. The fragments of monoclonal antibodies are cleared more rapidly from the circulation and may be good candidates for labelling with Tc-99m [275]. Labelling of monoclonal antibodies with Tc-99m by stannous chloride reduction does not produce a very stable complex [276]. Chelation of Tc-99m to DTPA-conjugated protein has been investigated [247,248], however, non-specific labelling of the protein by Tc-99m remains to be a problem. Paik *et al* [276] demonstrated that DTPA labelled monoclonal antibodies coupled with Tc-99m in the absence of free DTPA contains both

Figure 4. The Chemical Structures of New Lipophilic Chelates[274]

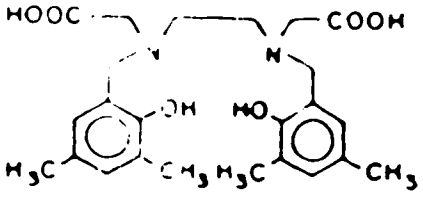
Me,HBED = N,N'-bis(2-hydroxy-3,5-dimethylbenzyl)ethylenediamine-N,N'-diacetic acid

t-butyl HBED = N,N'-bis(5-t-butyl-2-hydroxy-3-methylbenzyl)ethylenediamine-N,N'-diacetic acid

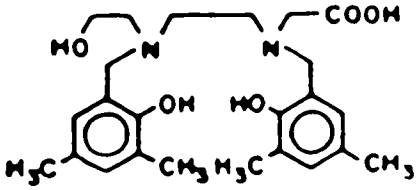
SHBED = N,N'-bis(2-hydroxy-5-sulfobenzyl)ethylenediamine-N,N'-diacetic acid

HMBA = N,N'-bis(2-hydroxy-3,5-dimethylbenzyl)ethylenediamine-N-(2-hydroxyethyl)-N' acetic acid

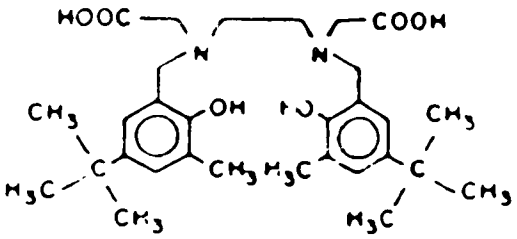
DPLED = N,N'-bis(5-deoxypyridoxyl)ethylenediamine-N,N'-diacetic acid



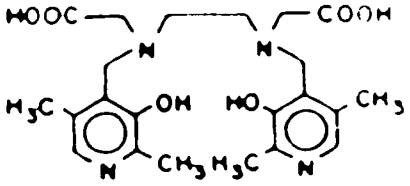
Me,HBED



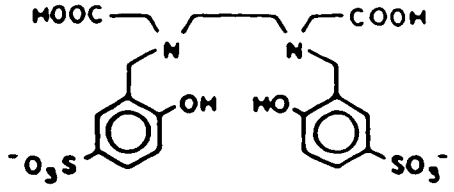
HBMA



t-butyl HBED



DPLED



SHBED

direct-labelled and chelated radioisotopes. Active research is now being conducted to find a chelate that will provide a stable Tc-99m labelled compound for *in vivo* use.

3. MATERIALS AND METHODS

3.1 Preparation of PNA Solutions

Affinity purified peanut agglutinin (PNA) was obtained in a lyophilized form from Sigma Chemicals (St Louis, Mo.). The PNA was reconstituted with 0.01M phosphate buffered saline (PBS) pH 7.1 to a concentration of 1 mg/ml for radioiodination studies. For labelling with Indium-111, the PNA was reconstituted with 0.2M phosphate buffer, pH 8.5 to give a concentration of 20 mg/ml. The reconstituted protein solution was passed through a 0.22μ Millipore® filter into a sterile Falcon® tube. The PNA solution was always freshly prepared for each use.

3.2 Quantitative Protein Analysis

The Bio-Rad® protein assay kit (Bio-Rad Lab, Miss., ⌣ used for protein concentration determinations. The assay utilizes the quantitative differential colour change of a dye, Coomassie Blue G-250 in response to different protein concentration. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The absorbance of the solutions were read at 595 nm using a Unicam Sp 1800 UV Spectrophotometer with a blank reference of the appropriate buffer used with the protein samples.

A standard curve of 10 to 1000 μg/ml protein concentration was generated using the supplied bovine gamma globulins as the protein standard. A similiar curve was also obtained by using PNA as the standard (see appendix 1). Hence, all further protein concentration determinations were performed using the supplied bovine gamma globulin as the standard. A new standard curve was generated everytime a protein concentration determination was made.

3.3 Radioiodination of PNA

3.3.1 Quality Control of Radioiodine Solution

Iodination grade I-125 and I-131 were obtained from the Edmonton Radiopharmaceutical Manufacturing Centre, Edmonton, Alberta. The radionuclidic purity of the radioiodine was determined by the gamma ray spectrum of each solution in a calibrated multichannel analyzer (Canberra Series 40 MCA) and compared to the published spectra.

Radiochemical purity was determined by ITLC with 85% methanol in water as the solvent system. About 20 KBq of each isotope solution were spotted on Gelman Silica Gel ITLC strips (20 cm in length) and allowed to air dry. The strips were then developed for about 15 cm in the solvent. The solvent front was marked and the strip was cut into 1cm segments and counted in a well type gamma detector (Beckman 8000 counter) to obtain a distribution of the radioactivity at the origin and at the solvent front.

3.3.2 Radioiodination Procedure

Radioiodination was carried out using the mild oxidizing agent Iodogen® (1,3,4,6-Tetrachloro-3α-6α diphenylglycouril, Pierce Chemicals, Rockford, IL.). Using a stock solution of 1 mg in 5 ml chloroform, 2 μg of iodogen were coated into the bottom of a 12x75mm culture tube for each 50 μg of PNA. The chloroform was evaporated under a gentle stream of nitrogen. The PNA solution (1 mg/ml in PBS) was added to the required amount of iodination grade NaI-125 (4000 MBq/ml in 0.1N NaOH) or NaI-131, which had been neutralized with 50 μl of 0.5M phosphate buffer. The mixture was transferred to the iodogen coated culture tube and was allowed to incubate for 45 minutes with occasional gentle agitation. At the end of the incubation period, the reaction mixture was transferred to another culture tube containing 20 μl of 1M NaI. The reaction mixture was allowed to sit for another 20 minutes and the free unreacted radioiodide was then separated from the radiolabelled protein by means of gel exclusion chromatography using a Bio-Gel® P6DG column (Bio-Rad

Lab., Miss., Ont.).

3.3.3 Separation of Unreacted Radioiodide

Bio Gele P6DG (Bio-Rad Lab, Miss., Ont.) is a spherical desalting polyacrylamide gel with an exclusion limit of about 6000 daltons and a hydrated particle size range of 90-18& μm. The gel was first allowed to hydrate overnight in PBS. Twice as much PBS was used as the expected packed volume (1 gram of dry gel will swell to about 8 ml of packed volume) . A 1.0x30 cm Econo Column (Bio-Rad Lab, Miss., Ont.) with about 15 cm of packed gel bed volume was used and this provided good separation of free radioiodide from the labelled protein when eluted with PBS.

The non-specific protein binding sites on the gel column were saturated by passing 0.5 ml of 1% BSA in PBS through the column before the application of the radiolabelled protein mixture. The reaction mixture was applied onto the column bed and allowed to drain in. A small volume of PBS was used to wash the mixture into the gel bed. The eluate was monitored for both UV absorbance by the protein and for radioactivity. A UV monitor (LKB 2238 UCICORD SII) was set at 280 nm wavelength and the baseline adjusted to zero by eluting with PBS. The eluate was also passed across the face of a shielded 3"x3" NaI(Tl) crystal detector coupled to a single channel analyzer. The elution profile displaying the absorbance at 280 nm and the radioactivity was recorded on an LKB 2210 dual pen recorder.

Aliquots of about 1 ml were collected as soon as the protein absorbance was detected by the recorder and the collection continued until the absorbance returned to baseline. The free radioiodine was collected as a single batch. All the fractions collected were measured for radioactivity in a Picker Isotope Dose Calibrator and the protein fractions with the bulk of the radioactivity were pooled, mixed well and used.

3.3.4 Quality Control Of Radioiodinated PNA

3.3.4.1 Trichloroacetic Acid (TCA) precipitation

Small aliquots of the purified labelled protein (about 2 to 5 μl) were diluted to 0.5 ml with 1% BSA in PBS in an Eppendorf® microcentrifuge tube and counted for radioactivity. Then, 0.5 ml of 25% TCA was added and the mixture was mixed well on a vortex mixer. After standing for 10 minutes, the mixture was centrifuged in an Eppendorf® centrifuge for 10 minutes. The precipitate was washed twice with 1 ml PBS with centrifugation. The washed pellet, as well as the combined washings were recounted for radioactivity and the percentage of added radioactivity which appeared in the pellet was calculated.

3.3.4.2 Instant Thin Layer Chromatography

A small spot of the purified radiolabelled protein was applied to a 20 cm Gelman Silica Gel ITLC strip and allowed to air dry. Each strip was then developed in 85% methanol in water system to about 15 cm in length. The strips were then cut into 1 cm segments and counted for radioactivity in a Beckman 8000 gamma counter.

3.4 Radiochelation with Indium-111

3.4.1 Preparation of In-111 Citrate Solution

Chelation grade In-111 chloride was obtained from Atomic Energy of Canada Ltd, Vancouver, B.C., Canada. The In-111 chloride was diluted with an equal volume of 0.1M citrate buffer pH 6.0 to produce the In-111 citrate. The In-111 citrate solution was further diluted with 0.1M citrate buffer pH 6.0 to obtain the specific activities desired.

3.4.2 Preparation of In-111 DTPA Solution

A 0.01M solution of DTPA (Aldrich Chem.) was made with 0.1N hydrochloric acid. Aliquots of the DTPA solution containing the required amount DTPA were pipetted into microcentrifuge tubes and In-111 chloride was then added to the DTPA solution and mixed well with a vortex mixer.

After 15 minutes, a small spot of the mixture was applied onto a silica gel plate and developed in 10% ammonium acetate/MeOH to assay for the completness of the chelation reaction.

3.4.3 Preparation of DTPA Anhydride Coated Vials

To each clean, dried, acid washed reactivial (Pierce, Rockford, Illinois) , an aliquot of a suspension of DTPA dianhydride (CalBioChem, La Jolla, CA.) in freshly distilled methylene chloride was added. The volume of the suspension in each vial was made up to 100 μl by adding freshly distilled methylene chloride. The methylene chloride was evaporated under a gentle stream of nitrogen gas in a fumehood leaving a thin coating of DTPA dianhydride on the wall of the reactivial. The DTPA coated vials were then capped and used immediately or stored at -80°C.

3.4.4 Reaction of PNA with DTPA Dianhydride

To the DTPA dianhydride coated reactivial, 100 μl of PNA solution (2 mg/100 μl in 0.2M phosphate buffer pH 8.5) was added. The reactivial was then capped and mixed on the vortex-mixer for 5 minutes.

The free hydrolyzed DTPA was removed from the reaction mixture by gel exclusion chromatography. Bio-Gel® P-200 was allowed to swell overnight in 0.01M citrate buffer pH 6.0. The gel slurry was poured into a 30 cm Bio-Rad® Econo-column (internal diameter 1.0 cm) to give a packed gel volume of about 16 to 17 cm. The reaction mixture was applied onto the column and eluted with 0.01M citrate buffer pH 6.0. The eluate was monitiored by a UV

spectrophotometer (LKB 2238 UVICORD SII) set at 280 nm wavelength. Under these conditions, with a flow rate of about 5 ml/hour, the DTPA labelled protein usually eluted within 45-60 minutes while the free hydrolyzed DTPA appeared at 2 to 3 hours. The protein fraction corresponding to monomeric PNA was collected.

After determination of protein concentration using the Bio-Rad protein assay kit, aliquots containing 100 μg or 20 μg of PNA were pipetted into clean, acid-washed Eppendorf® micro-centrifuge tubes. The aliquots were then freeze dried overnight resulting in a kit-form preparation containing purified DTPA-PNA.

3.4.5 Chelation of DTPA-PNA with In-111 Citrate

Chelation grade In-111 chloride as obtained from Atomic Energy of Canada, Vancouver, was diluted with a equal volume of 0.1M citrate buffer pH 6.0 before use. Routinely, 25-100 μl of the diluted In-111 citrate were added directly to the freeze-dried DTPA-PNA. The reaction was allowed to proceed for 15 minutes with occasional mixing using the vortex-mixer.

3.4.6 Purification Using Minicolumn Centrifugation

Bio-Gel® P-100 was allowed to hydrate overnight in 0.1 M citrate buffer pH 6.0. A 2.5 ml aliquot of the gel slurry was poured into a polystyrene minicolumn (Serva, New York). The minicolumn was centrifuged for 15 minutes at 2500 rpm to remove the excess buffer in the gel.

The chelation reaction mixture was added to the exposed gel surface of the minicolumn. The minicolumn was then centrifuged for 15 minutes at 2500 rpm; 100 μl of 0.1M citrate buffer added to the gel surface and the minicolumn recentrifuged for another 15 minutes. The eluate was collected in a Eppendorf® microcentrifuge tube and was assayed for total radioactivity in an isotope dose calibrator.

3.4.7 Quality Control of In-111 Radiochelated PNA

The radiochemical purity of the In-111 radiochelated PNA was determinated by thin layer chromatography. A small spot of the purified protein solution was applied onto a 1.5x8.0 cm cellulose TLC plate (Eastman Chromagram Sheet, Rochester, N.Y.) . The plate was developed in 0.1N HCl and methanol (30/70). After development, the plate was allowed to air dry and the distribution of the radioactivity on the plate was determination by a Berthold linear TLC scanner (Berthold Lab., Germany) coupled to a Canberra Series 40 multichannel analyzer (MCA).

3.5 Study on the Radiochelation Procedure

3.5.1 The Effect of pH on Conjugation Efficiency

PNA solutions were prepared in 0.2M phosphate buffer at various pH, ranging from 5.4 to 9.8. To a DTPA dianhydride coated reactivial, 100 μl(2 mg) of the PNA solution was added and mixed for 5 minutes using the vortex-mixer. A small aliquot of the reaction mixture was mixed with a tracer amount of In-111 citrate for 15 minutes with occasional mixing. A small spot was then applied on a cellulose TLC plate and developed in 0.1N HCl and methanol (30/70). The percent conjugation efficiency obtained at each pH value was calculated from the distribution of the radioactivity on the TLC plate.

3.5.2 Effect of PNA Concentration on Conjugation Efficiency

PNA was dissolved in 0.2M phosphate buffer pH 8.5 to give various PNA concentrations ranging from 1 to 20 mg/ml. The required amount of DTPA dianhydride to achieve a 10:1 DTPA:PNA ratio at each concentration of PNA was coated onto different reactivials for the chelation reaction. To the DTPA dianhydride coated reactivial, 100 μl of the PNA solution was added and mixed for 5 minutes using the vortex-mixer. A small aliquot of the reaction mixture was then mixed with a tracer amount of In-111 citrate, and the efficiency

of the conjugation reaction was measured by TLC.

3.5.3 Effect of the Starting Ratio of PNA to DTPA Dianhydride on Conjugation Efficiency

A fixed amount of PNA solution (2 mg in 100 μl) in 0.2M phosphate buffer pH 8.5 was added to reactivials containing various amount of DTPA dianhydride, resulting in DTPA to PNA ratios ranging from 1:1 to 50:1. The mixture was agitated on a vortex-mixer for 5 minutes. A tracer amount of In-111 was added to an aliquot of the PNA-DTPA sample and TLC was performed to measure the extent of radiolabelling.

3.6 Characterization of Radiolabelled PNA

3.6.1 *In Vitro* Studies

3.6.1.1 Radiochemical Stability of In-111 DTPA-PNA

The radiochemical stability of In-111 labelled PNA was checked by means of TLC. The purified radiolabelled protein solution was stored at 4°C and a small aliquot of radiolabelled protein solution was taken from the stock solution at various time intervals from 1 to 72 hours and applied onto a cellulose TLC plate. After developing the plate in 0.1N HCl/MeOH (30/70), the distribution of the radioactivity on the TLC plate was measured by scanning on the Berthold linear TLC scanner coupled to a multichannel analyzer.

3.6.1.2 Challenging with Chelating Agents

The stability of In-111 DTPA-PNA against transchelation to free chelating agents in solution was assessed by means of TLC. An aliquot of the radiochelated PNA (20 μg, 4 MBq) was incubated with either a 1:1 or 10:1 molar ratio of DTPA or EDTA at 37°C in an Eppendorf® tube. At various time intervals, a small aliquot of the mixture was spotted onto a cellulose TLC plate and developed in 0.1N HCl/MeOH (30/70). The

amount of radioactivity at the origin and at the solvent front was assessed by scanning the TLC strip on the linear TLC radioactivity scanner.

3.6.1.3 Challenging with Serum

To determine whether transchelation of the In-111 from PNA to serum may occur readily, a small aliquot of the radiochelated PNA was incubated with 1 ml of fresh rabbit serum at 37°C in an Eppendorf® tube . At various time intervals, 50 μl of the solution were removed from the Eppendorf® tube and incubated with 10 mg of T-Synsorb (ChemBioMed, Edmonton, Alberta) which was previously washed with 1% BSA to saturate any non-specific protein binding sites. After 8 hours of incubation, the T-Synsorb was centrifuged for 5 minutes in an Eppendorf® centrifuge, washed twice with 1 ml PBS and recentrifuged. The T-Synsorb and the combined washings were then counted for radioactivity in the Beckman 8000 Gamma Counter.

3.6.1.4 Studies with Neuraminidase-Treated Human Red Blood Cells

"O" group blood, freshly collected from normal heathy donors, was kindly donated by the Canadian Red Cross Society Blood Transfusion Service. The T-antigen sites on the surface of the normal red blood cells were exposed by treating with neuraminidase. One ml of whole blood was washed 3 times with 10 ml of PBS. Packed RBC (100 μl) were removed from the test tube and incubated with 1 ml (1 unit/ml) of Clostridium perfringens neuraminidase (Sigma Chemicals, St. Louis, Mo.) and 2 ml of PBS for 45 minutes at 37°C. Control RBC were treated with PBS alone. The RBC were then washed 4 times with 10 ml of 5% Fetal Calf Serum (FCS) in PBS. Fifty microlitre of packed RBC from each treatment were diluted in 16.67 ml of PBS to give a final concentration of 0.3% (v/v).

The binding of I-125 PNA, In-111 DTPA-PNA, In-111 citrate and In-111 DTPA to neuraminidase treated red blood cells (N'RBC) was studied by mixing aliquots of the tracers with 500 μl of the N'RBC. The tracer solutions and the N'RBC were mixed in

BSA-precoated Eppendorf tubes with 500 μl of PBS or 0.05M D-galactose in PBS (for galactose inhibition study). The mixture was allowed to incubate for 45 minutes on ice with occasional gentle agitation. The mixture was then centrifuged for 10 minutes at 16000G in an Eppendorf® centrifuge. The RBC were washed 3 times with 1 ml PBS with centrifugation. The cell pellet as well as the combined washings were counted for radioactivity.

3.6.1.5 Binding to T-Synsorb

T-Synsorb (ChemBioMed, Edmonton, Alberta) is a synthetic immunoadsorbent containing the immunodominant structure β-D-galactosyl (1→3) α-N-acetyl D-galactosamine. Ten mg aliquots of T-Synsorb were incubated with 1 ml of 1% BSA for 6 hours at room temperature to saturate the non-specific binding sites. The BSA was removed and the synsorb washed twice with 1 ml PBS. A small aliquot of the diluted lectin (I-125 and In-111 labelled) was added to the synsorb with 500 μl of PBS or 0.05M D-galactose in PBS (for inhibition study). The mixture was allowed to incubate for 8 hours with gentle mixing by inversion on a Lab-Quake rotomixer (Labindustries, Berkeley, Calif.). The mixture was counted for total radioactivity and then centrifuged for 5 minutes in an eppendorf® centrifuge, washed 3 times with 1 ml PBS and the pellet recounted for radioactivity.

3.6.1.6 Binding to Tumor Cells

Various tumor cell lines were incubated with radiolabelled PNA to determine the binding specificity of this protein. T-antigen expressing TA_3/Ha adenocarcinoma tumor cells, TA_3/St and RI lymphoma cell line, as well as the non-T expressing EL_4 tumor cell line were used. The various cells used were maintained in culture in RPMI 1640 medium (Gibco) supplemented with heat denaturated 10% fetal calf serum and 2 mM L-glutamine. The cells were taken from the log phase of growth and greater than 95% excluded trypan blue. Aliquots of 10^7 cells from each of the cell lines were incubated with

the various tracers including I-125 PNA, In-111 DTPA-PNA , In-111 citrate and In-111 DTPA. The amount of lectin used for incubation was 20 ng. After incubation for 45 minutes on ice, the cells were centrifuged for 5 minutes in the Eppendorf® centrifuge and washed twice with 1 ml PBS. The cell pellets and the combined washings were counted for radioactivity. D-galactose at a concentration of 0.05M was added to the mixture to demonstrate the specificity of the binding.

3.6.2 *In Vivo* Studies

3.6.2.1 Animal Tumor Model

Male CAF_1/J mice weighting approximately 20 g were used for biodistribution and whole body imaging studies. TA_3/Ha cells in tissue culture (about 10^6 cells) were injected intraperitoneally into CAF_1/J mice. After 10 days, the ascites fluid was collected, concentrated by centrifugation and the cells counted on a microscope hemocytometer grid. The T-antigen secreting TA_3/Ha mammary adenocarcinoma model was produced by subcutaneous (s.c.) innoculation with the TA_3/Ha tumor cells. Aliquots of 10^6 viable TA_3/Ha tumor cells from ascites were injected s.c. in the right flank and allowed to grow in the mice for 10-14 days. This resulted in well vascularized, solid tumors of approximately 150 to 200 mg size.

All the mice used in the various studies were maintained on standard laboratory food and free access to tap water. No pretreatment with 0.01% KI had been performed in order to show the distribution of free radioiodide after deiodination of the radiolabelled PNA had occurred.

3.6.2.2 Biodistribution Studies

The biodistribution of I-125 PNA, In-111 DTPA-PNA, I-125 DTPA-PNA and In-111 citrate in male CAF_1/J mice was studied.

Aliquots of 0.1 ml of the labelled protein (1 μg PNA) or tracer solution containing about 80 KBq were injected via the tail vein. Monojecto syringes were used to minimize the residual volume in the syringe, The doses in the syringes were assayed for radioactivity in a NaI(Tl) detector coupled to a Canberra series 40 MCA both before and after the injection to standardize the actual amount of activity that has been injected. For dual label study, the iodinated PNA and indium-chelated PNA were prepared separately and mixed just prior to the injection to give approximately 110 KBq radioactivity for each labelled protein.

Three extra doses were drawn up in syringes, the contents emptied into Fisher counting vials and diluted 1 to 100 . Aliquot samples of 100 μl, 200 μl and 300 μl were taken from each dilution and counted as injection standards to correlate the injection dose to the amount of radioactivity in the samples.

At various time intervals, the mice were sacrificed by cardiac puncture under CO_2 anesthesia. The blood, tissues of interest and the remaining carcass were collected and weighted in tared Fishere counting vials. The samples were then counted in a programmable NaI(Tl) gamma well (Beckman 8000 Gamma Counter) together with the injection standards. The coincidence method of counting was used to determine the absolute counts for I-125. For dual label study with In-111 and I-125, the spillover counts from In-111 into the I-125 channel were calculated and appropriate corrections were made.

3.6.2.3 Urinary Excretion of PNA in the Rat

Because of the small volume of urine available from mice, the urinary excretion of radiolabelled PNA was studied in the rat. Sprague-Dawley rats weighting approximately 150 g were injected with about 17 MBq of In-111 DTPA-PNA (25 μg) via the tail vein. The rats were maintained in a metabolic cage for urine collection. The first batch of urine was collected 12 hours post injection, and the final batch collected at 30 hours post injection. The urine was subjected to gel filtration chromatography with

Bio-Gele P-200 using a 1.0x50 cm Bio-Rade Econo-column and eluted with PBS. The eluate was monitored for both UV absorbance at 280 nm and for In-111 radioactivity. The protein peaks which were associated with radioactivity were collected separately and an aliquot was used for incubation with 10 mg of T-Synsorb to determine the retention of biological activity of the excreted PNA.

3.7 Dynamic Kidney Activity Time Curve

Normal male New Zealand White (NZW) rabbits were used to study the renal dynamics of intravenously injected radiolabelled compounds. The rabbits were anesthetized with an I.M. injection of 50 mg/kg ketamine (Rogar/STB, Montreal, Quebec) and 10 mg/Kg xylazine (Cutter Laboratory, Miss, Ont.) and placed supine on a Searle PH0 Gamma IV Gamma Camera detector interfaced to the ADAC CAM II Aquisition Unit. The low energy parallel hole collimator was used for In-111 data collection (247 KeV), while the high energy parallel hole collimator was used for I-131 data collection.

Each of the injection syringes containing about 3 MBq of each of In-111 DTPA-PNA, In-111 citrate, In-111 DTPA or I-131 PNA were imaged and the data stored on the floppy disc for subsequent calculation of the count rate in the injection dose.

The compounds were injected via the lateral ear vein, and dynamic data were collected and stored at an aquisition rate of 20 second frames for 10 minutes, followed by 1 minute frames for 60 minutes (128x128 matrix). A static image of the syringe with residual radioactivity was also taken after the dynamic study. At various time intervals post injection, static images of the rabbit were also obtained to determine the elimination of the compound from the organs of interest.

The uptake and elimination curves were reconstructed on the computer, and regions of interest drawn around the two kidneys, the bladder, the liver and a background region distal to the left kidney. The background corrected activity time curve for each organ of interest was plotted from the data and corrected to the injection dose. The static images were used to

determine the percent activity remaining in each organ at various times post injection.

3.8 Whole Body Scintigraphic Study

3.8.1 Mice

Male CAF_1/J mice weighting approximately 20 g bearing a well defined solid TA_3/Ha tumor on the right flank were used for whole body scintigraphic studies. About 2 MBq (4 μg) of I-125 PNA or 3 MBq (2 μg) of In-111 DTPA-PNA were injected via the tail vein. The mice were anesthetized with 1.3 mg ketamine and 0.13 mg xylazine intraperitoneally and were held in a prone position on a positioning board by means of masking tape. Serial scintigraphic images at 6 hr, 24 hr, 48 hr and 72 hr post injection were collected on a Searle PH0/ Gamma IV camera (100,000 counts, pinhole collimator, 5mm aperture) and stored on a ADAC CAM II clinical acquisition processing unit.

3.8.2 Rabbit

Normal healthy NWZ rabbit were anesthetized with an intramuscular injection of 50 mg of ketamine. About 7.5 MBq of In-111 DTPA-PNA (10 μg) were injected via the lateral ear vein and serial scintigraphic images were taken at various time interval post injection. 100,000 counts were collected for each image on the Searle Gamma Camera using a parallel hole low energy collimator and stored on the ADAC CAM II Computer.

4. RESULTS AND DISCUSSION

4.1 Radioiodination of Peanut Lectin

The radionuclidic and radiochemical purity of the radioiodination grade I-125 and I-131 sodium iodide solutions, obtained from Edmonton Radiopharmaceutical Centre, were assessed prior to use in radioiodination procedures. The radionuclidic purity was determined by obtaining the gamma ray spectra of a small aliquot of the radioiodide solution with a multichannel analyzer coupled to a 3" x 3" sodium iodide (Tl) well crystal. The spectra obtained were found to correspond with those of published spectra for these radionuclides in the literature[122]. Radiochemical purity of the radioiodide solutions was assessed by means of ITLC. About 20 KBq of the radioiodide solution were applied onto a silica gel ITLC strip and developed in an 85% aqueous methanol system. Under this solvent system, sodium iodide migrated with the solvent front (Rf value 1.0) while other oxidized forms of iodine such as sodium iodate remained at the origin. High radiochemical purity (>98%) was routinely demonstrated on both the I-125 and I-131 radioiodide solutions obtained. On the basis of these two tests, the radioiodide solutions obtained were deemed to be of high radionuclidic and radiochemical purity and suitable for subsequent radioiodination procedures.

Lyophilized affinity-purified PNA was reconstituted with 0.01M PBS to give a final concentration of 1 mg/ml. The PNA solution was mixed with the radioiodide solution buffered with 50 μl of 0.5M phosphate buffer pH 7.2. The iodination reaction was initiated by transferring the protein radioiodide mixture to an Iodogen® coated glass culture tube. For every 50 μg of PNA to be iodinated, 2 μg of Iodogen® were used. The amount of Iodogen® used has been shown not to significantly affect the biological activity of a number of proteins[242]. A particular advantage with the Iodogen® technique is that no final reduction step is required as the reaction is terminated simply by removal of the contents from the Iodogen® coated tube.

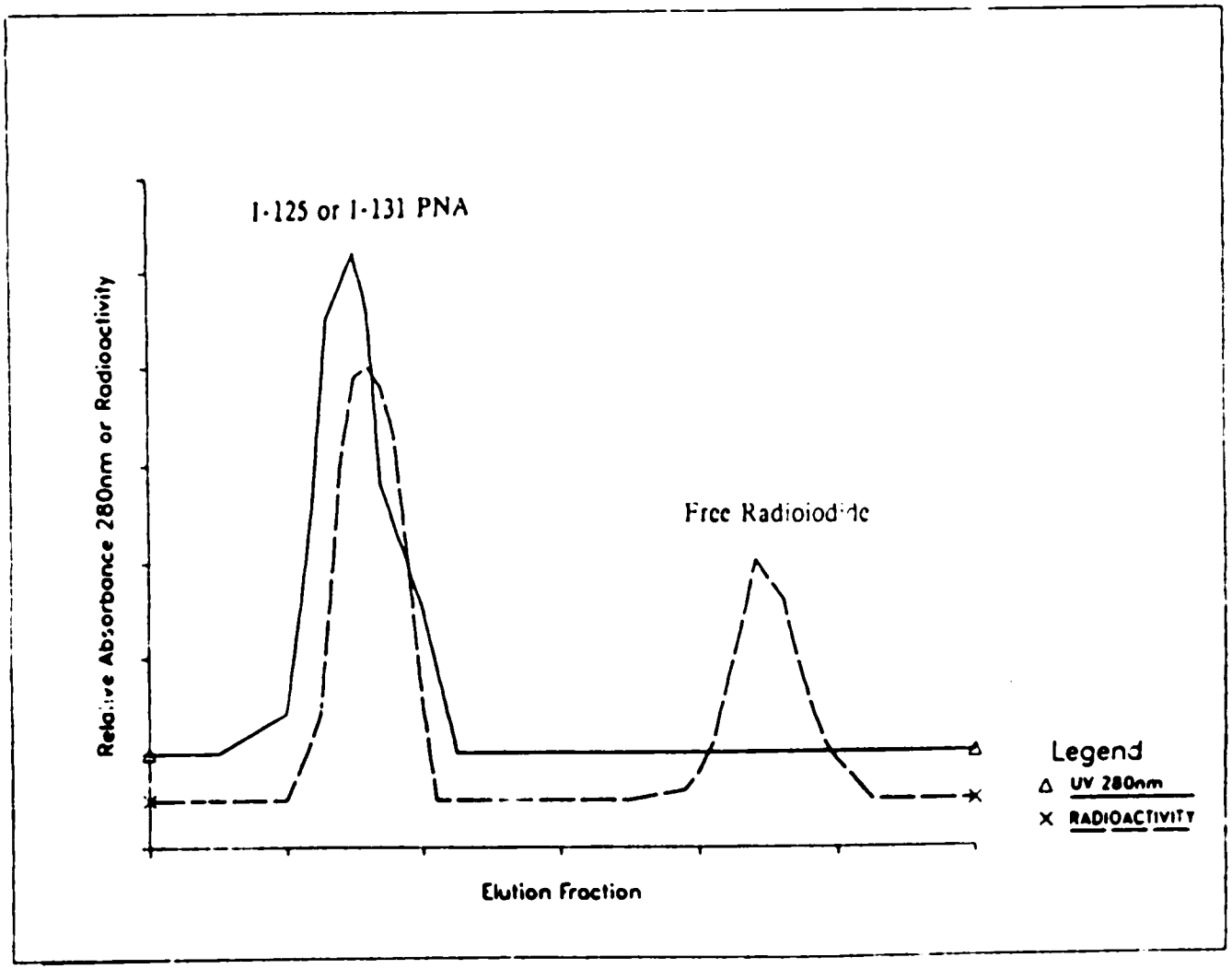
The PNA was radioiodinated to a specific activity of approximately 110 MBq/μg for the biodistribution studies in mice and to approximately 740 MBq/μg for radioimaging studies. The specific activity of the PNA was regulated by the amount of radioiodide solution used. With increasing amount of radiodide, the specific activity of the radioiodinated PNA increased while the radiodination efficiency decreased. A radioiodination efficiency of about 40 to 50% was usually obtained when the specific activity of the radioiodinated PNA was low, while approximately 20% efficiency was obtained for high specific activity labelling.

The free radioiodide in the reaction mixture was separated from the radioiodinated PNA by gel exclusion chromatography. Bio-Gel® P6DG, with a molecular weight exclusion limit of approximately 6000 daltons, was used for the separation. With a gel bed height of about 15cm, good separation was obtained by elution with PBS. Figure 5 illustrates a typical plot of radioactivity and UV absorbance of the I-125 PNA reaction mixture when it is passed through a Bio-Gel® P6DG column. The radioiodinated PNA appears at the void volume while the free radioiodide elutes at about 5 ml after the labelled protein. Quality control of the radioiodinated PNA routinely indicated better than 98% radiochemical purity, using both ITLC and TCA precipitation.

4.2 Radiochelation with In-111

One of the major concerns with a radiometal chelation reaction is the interference by trace metals in the reaction vessels or the buffers used [168]. To minimize this problem, all glassware used in this project were soaked in mixed acid (HNO_3/H_2SO_4 : 50/50) for 1 hour and rinsed thoroughly with double distilled deionized water. The buffers used were passed through a Chelex-100® (Bio-Rad Lab., Miss., Ont.) column to remove the trace metal contamination. Metal-free pipette tips (Bio-Rad Lab., Miss., Ont.) were also used to minimize the introduction of any trace metals.

Figure 5. Separation of Free Radioiodide from I-125 or I-131 PNA by Bio-Gele P6DG Chromatography



4.2.1 Conjugation Reaction of PNA with DTPA Dianhydride

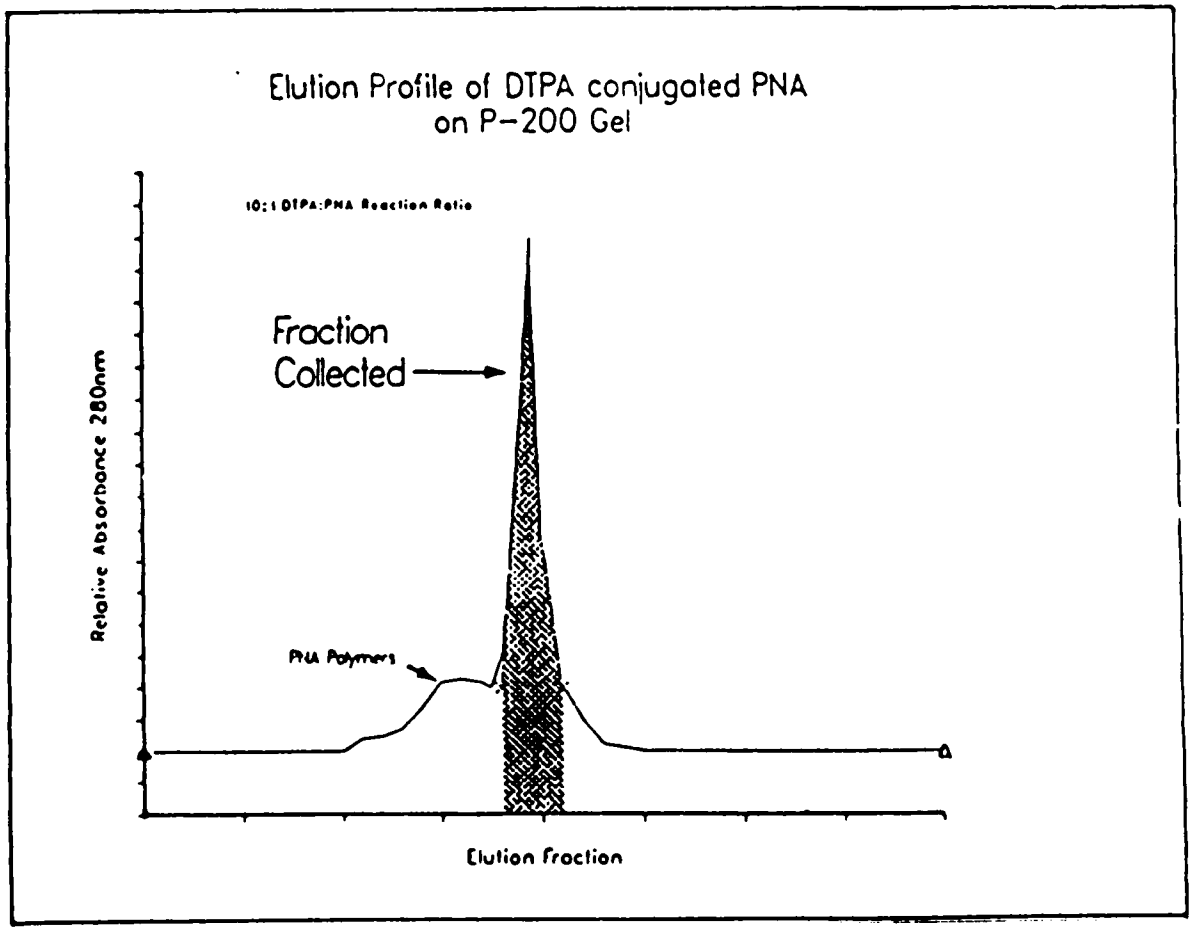
PNA at a concentration of 20 mg/ml was added to the DTPA dianhydride coated reactivials. DTPA dianhydride reacts with free amino groups, mainly lysine residues, on the PNA molecule. Any excess DTPA anhydride which did not react with PNA will be hydrolyzed to free DTPA[266].

After the conjugation reaction, the free hydrolyzed DTPA was removed from the DTPA-conjugated PNA by means of gel exclusion chromatography. Since there are two protein reactive groups on each of the DTPA anhydride molecule, dimerization of PNA may occur. Any high molecular weight aggregates of PNA will also be separated from the monomeric PNA during the same step. Bio-Gel P-200 (exclusion limit 200,000 daltons) was used for the separation and 0.01M citrate buffer pH 6.0 was used as the eluant. Under the conditions used, with a column dimension of 1 cm I.D. X 20 cm height, and at a flow rate of 5 ml/hour, the high molecular weight aggregates of DTPA-PNA eluted at the void volume, followed by the monomeric PNA fraction at about 45-60 minutes. The free hydrolyzed DTPA appeared at about 2 to 3 hours. The protein fraction corresponding to monomeric PNA was collected (see figure 6). Less than 10% of high molecular weight form of PNA was usually observed after the conjugation reaction.

The concentration of PNA in the eluate was determinated by Bio Rad Protein Assay Kit®. The standard curve was generated by using Bio-Rad Protein Standard I (Gamma Bovine Albumin) and by using PNA itself. Both standard curves were very similiar thus the Bio Rad Protein Standard I was used in subsequent studies. A new standard curve was generated everytime a protein concentration determination was made. (Appendix 1 illustrates a PNA concentration verus absorbance at 595 nm wavelength for each of the standards)

Aliquots corresponding to 100 μg or 20 μg of DTPA-coupled PNA were pipetted into clean acid-washed Eppendorf® microcentrifuge tubes and were freeze dried overnight in a Labconco® freeze dryer. The 100 μg preparation was intended for use in animal biodistribution studies while the 20 μg preparation was used for radioscintigraphic studies. The freeze dried

Figure 6. Elution Profile of DTPA-Conjugated PNA on Bio-Gel® P-200



DTPA-PNA served as a convenient kit preparation which could be used immediately or stored at -4°C for use at a later time. The radiolabelling can be achieved by the simple addition of the appropriate amount of radiometal solution to the DTPA-PNA kit.

4.2.2 Radiochelation of In-111 to DTPA Coupled PNA

In-111 chloride, was routinely obtained from Atomic Energy of Canada as a no-carrier-added solution of approximately 220 MBq/350 μl in 0.1N HCl. The gamma ray spectrum from a small aliquot of the sample was obtained using a Canberra series 40 multichannel analyzer coupled to a 3" x 3" NaI crystal. The spectra obtained yielded main gamma peaks at 173 and 247 KeV corresponding to the published spectra of In-111 in the literature thus confirming the radionuclidic purity of the In-111 received[322].

The radiochelation reaction was usually performed within 1 to 2 days after the arrival of the radioisotope to take advantage of the high specific activity available. As In-111 radiodecay proceeds, the proportion of In-111 ions to other trace metallic contaminants will decrease and this will adversely affect the efficiency of the chelation reaction. The In-111 chloride was first diluted with an equal amount of 0.1M citrate buffer pH 6.0 before use. At a 10:1 starting DTPA anhydride to PNA molar ratio, the following radiolabelling efficiencies were obtained (table 15).

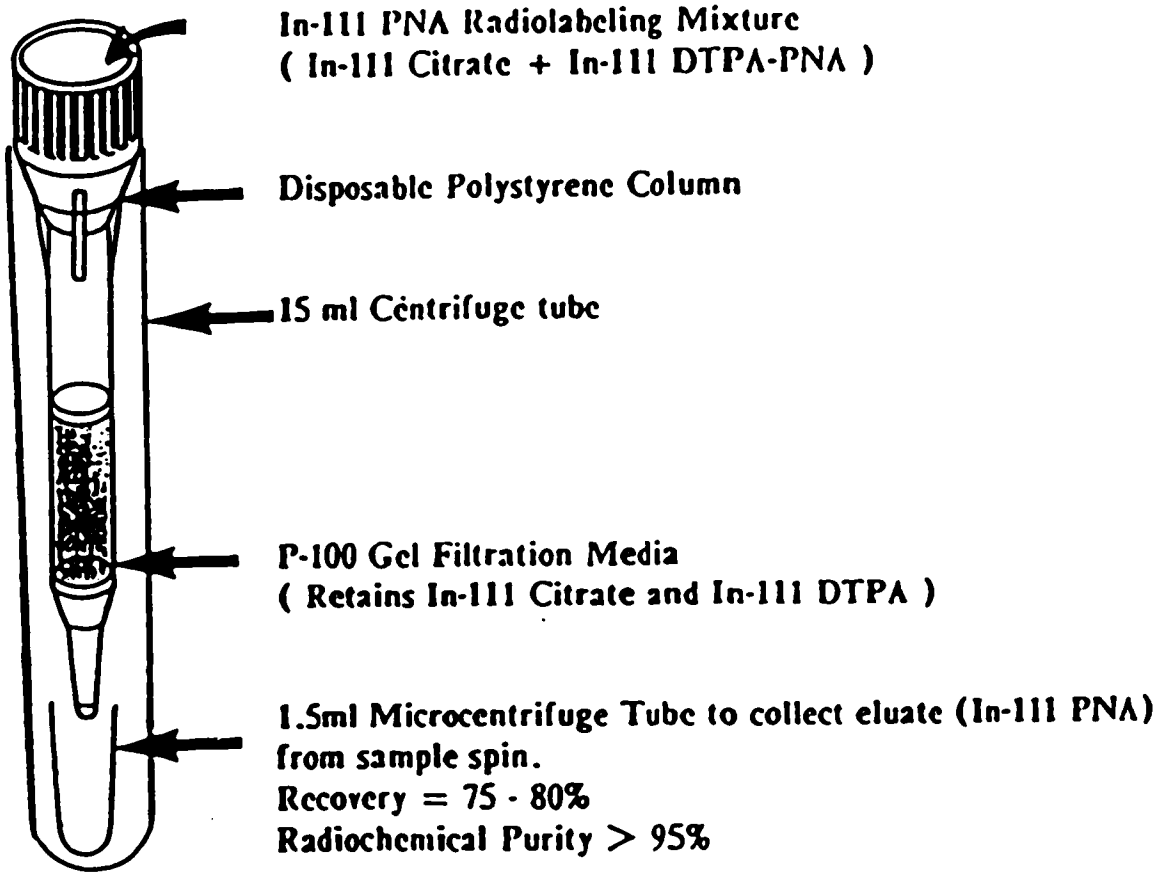
Under optimal conditions, labelling efficiencies in excess of 90% were usually obtained resulting in In-111 labelled PNA at a specific activity of approximately 150 to 185 MBq/μg. Higher specific activities can be obtained by increasing the amount of In-111 citrate in the reaction but will result in lower chelation efficiencies. Utilizing this radiochelation method, specific activity as high as 1.2 MBq/μg are obtainable for radioscintigraphic studies.

The In-111 labelled PNA was purified by the minicolumn centrifugation method[243]. Figure 7 shows the various components of the minicolumn apparatus. The method is fast, simple to perform, causes minimial dilution of the sample and has a high protein recovery. The minicolumn was filled with about 2.5ml of Bio-Gel P-100 (preswollen overnight in 0.1M

Table 15. Radiochelation efficiencies for In-111 labelling of DTPA-PNA.

Amount of PNA	Amount of In-111 citrate added	% radiochelation efficiency	specific activities obtained
100μg	1.11 MBq (40μl)	92.50%	181 KBq/μg
100μg	1.70 MBq (40μl)	93.45%	159 KBq/μg
100μg	2.09 MBq (45μl)	90.87%	189 KBq/μg
100μg	2.22 MBq (50μl)	91.72%	203.5 KBq/μg
100μg	1.58 MBq (30μl)	93.62%	147.6 KBq/μg
100μg	59.2 MBq (120μl)	63.50%	377.4 KBq/μg
20μg	68.86 MBq (140μl)	35.40%	1.22 MBq/μg
20μg	62.2 MBq (142μl)	30.60%	951 KBq/μg

Figure 7. Minicolumn Apparatus for Purification of In-111 DTPA-PNA



citrate buffer pH 6.0) and spun for 15 minutes at 2500 rpm in a Dynaco table top centrifuge to remove the excess buffer. The PNA reaction mixture was applied to the column and spun for 15 minutes at the same speed. A 100 μl wash of 0.1M citrate buffer was then applied to the column to increase the protein recovery. The amount of protein recovered was calculated from the amount of radioactivity collected in the eluate and the percentage of radioactivity that was bound to the protein. The quality and recovery of the PNA is affected by the speed of the centrifugation and the period of time spend in spinning the column. Table 16 summarizes the conditions used in the centrifugation method and the quality of the corresponding product. Usually about 80 to 85% of In-111 DTPA-PNA can be recovered from the minicolumn under the conditions routinely employed.

4.3 Radiochemical Purity of In-111 DTPA-PNA

The radiochemical purity of the In-111 labelled PNA was measured by thin layer chromatography. A small spot of the labelled PNA was applied onto a 2.5 x 10 cm cellulose TLC strip. The strip was then developed in 0.1N HCl/MeOH (30/70) to a height of 8 cm. Under this solvent system, the In-111 DTPA-PNA has a Rf value of 0.0-0.1 while the free In-111 citrate will travel to a Rf value of 0.7 - 0.8. The In-111 DTPA-PNA obtained after minicolumn centrifugation routinely gave higher than 98% radiochemical purity when tested in this TLC system.

4.4 Studies on the Radiochelation Reaction with In-111

Chelation of radiometalic ions via the use of bifunctional chelating agents is an alternative to radioiodination for radiolabelling of proteins. In-111 is a commonly used isotope for radiochelation reactions due to its easy availability, suitable gamma energies for radioscintigraphy and a convenient half-life. The DTPA-dianhydride technique has gained widespread acceptance for use in the radiolabelling of a number of proteins, especially antibodies for use in the radioimmunodetection of tumors. The dianhydride of DTPA reacts

Table 16. Amount of PNA Protein Recovery During Minicolumn Centrifugation Method.

Speed (RPM)	Time (min.)	% protein bound radioactivity (n=2)	% protein recovery (n=2)
1000	5	90.5 - 91.2	18 - 20
	10	93.4 - 94.6	22 - 25
	15	94.7 - 95.8	26 - 29
	20	96.5 - 96.7	29 - 31
	30	96.5 - 97.8	33 - 35
2000	5	98.5 - 98.7	42 - 44
	10	98.6 - 98.7	56 - 60
	15	98.8 - 98.8	60 - 62
	20	98.7 - 98.8	65 - 69
	30	96.4 - 97.2	85 - 88
2500	5	98.4 - 98.9	46 - 50
	10	98.7 - 99.0	77 - 80
	15	98.7 - 99.1	87 - 88
	20	97.1 - 98.5	88 - 89
	30	96.5 - 97.2	88 - 90
	60	94.2 - 94.6	88 - 90

with free amino groups on the protein molecule, especially with lysine residues to form a stable amide linkage[266-269]. Thus the efficiency of the coupling reaction between DTPA anhydride and the protein will be affected by the availability and the number of lysine residues in the protein molecule. The difference in amino acid compositions between PNA and the antibodies may significantly alter the efficiency of the attachment of the chelate. PNA contains only about 40 lysine residues per molecule, thus this will limit the efficiency of the incorporation of the DTPA residue and the absolute amount of DTPA that can be attached to the PNA molecule[166]. It was therefore desirable to study the optimal conditions for the radiochelation of PNA.

The coupling reaction between DTPA dianhydride and protein has been studied extensively by Hnatowich *et al* [266-268]. Several variables in the conjugation reaction had been shown to significantly affect the efficiency of the reaction. These included the pH of the buffer used, the protein concentration employed during the reaction and very importantly, the molar ratio of DTPA dianhydride to the protein used[266-268]. All of these factors were studied in this project with PNA.

4.4.1 The Effect of the pH of the Buffer

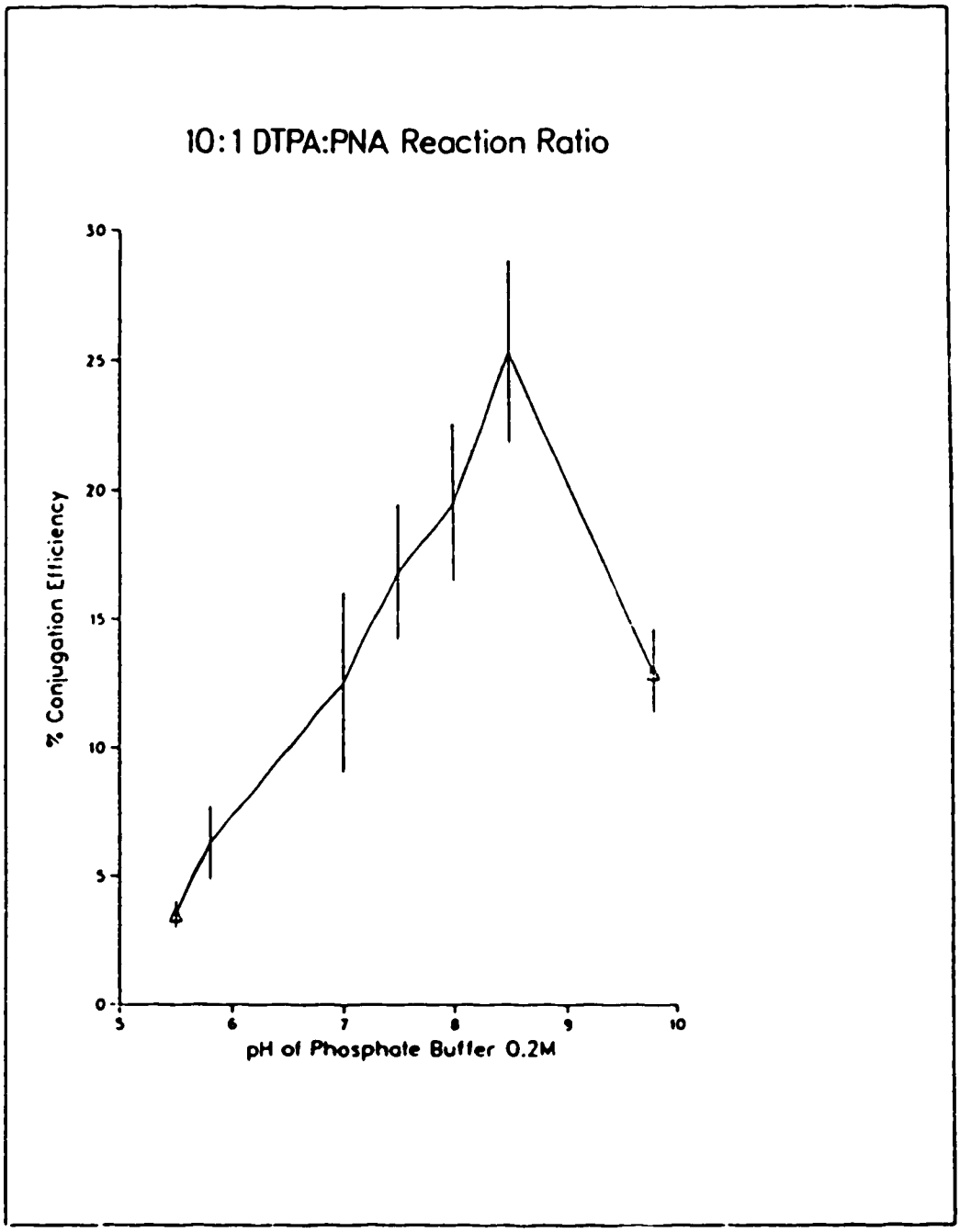
The pH of the buffer used had a significant effect on the efficiency of the coupling reaction between the DTPA and PNA. There is usually an optimal pH range, above and below which, the coupling efficiencies decreases rapidly[266]. The effect of pH of 0.2M phosphate buffer on the conjugation of DTPA to PNA is presented in table 17 and summarized graphically in figure 8.

The highest coupling efficiency was achieved at pH 8.5. This indicated the importance of the neutral amino groups of lysine residues during the conjugation reaction[277]. A rather high capacity buffer is also required since the hydrolysis reaction of the anhydride will result in the formation of acetic acid molecules, thus lowering the pH of the buffer[277]. Based on the results of this study, 0.2M phosphate buffer pH 8.5 was used during the subsequent coupling

Table 17. The Effect of pH on the PNA-DTPA Conjugation Reaction

pH of 0.2M PO_4 Buffer	% Conjugation Efficiency ± S.D. (n=3).
5.5	3.50% ± 0.56
5.8	6.30% ± 1.44
7.0	12.50% ± 3.58
7.5	16.80% ± 2.69
8.0	19.47% ± 3.13
8.5	25.30% ± 3.58
9.8	12.90% ± 1.78

Figure 8. The Effect of pH on the Conjugation Reaction Between PNA and DTPA Dianhydride



reaction between DTPA dianhydride and PNA.

4.4.2 The Effect of the Concentration of PNA

The efficiency of the conjugation reaction between proteins and DTPA is readily affected by the concentration of protein employed during the reaction[266,267]. Table 18 and figure 9 present the effect the PNA concentration has on the conjugation reaction. The conjugation efficiencies ranged from about 4% at 1 mg/ml PNA concentration to about 28% at a 20 mg/ml PNA concentration. Higher PNA concentrations may further increase the efficiency of the reaction but difficulty in solubilizing the lectin becomes a problem. Based on these observations, a 20 mg/ml concentration of PNA was chosen for use in subsequent studies.

The efficiency of the DTPA-dianhydride coupling reaction is determined by two factors, the interaction of the dianhydride with the amino groups on the protein, thus forming a stable protein conjugate, and the hydrolysis of the anhydride yielding free hydrolyzed DTPA[277]. Also among the reactive groups on the protein, the acylated products of the sulfhydryl moiety of cysteine and the imidazole group of histidine are very unstable, and the *O*-acylated hydroxyl group of tyrosine also hydrolyses readily[277]. At a low PNA concentration, the hydrolysis of the anhydride dominates and a low PNA coupling resulted. At a higher PNA concentration, the chance of the anhydride reacting with a PNA molecule before hydrolysis takes place increases, thus resulting in an overall increase in the coupling efficiency.

Similiar studies were performed by Hnatowich *et al* [266-268] on the optimal reaction conditions for labelling proteins and antibodies. In all of the studies, a higher protein concentration resulted in higher conjugation efficiencies.

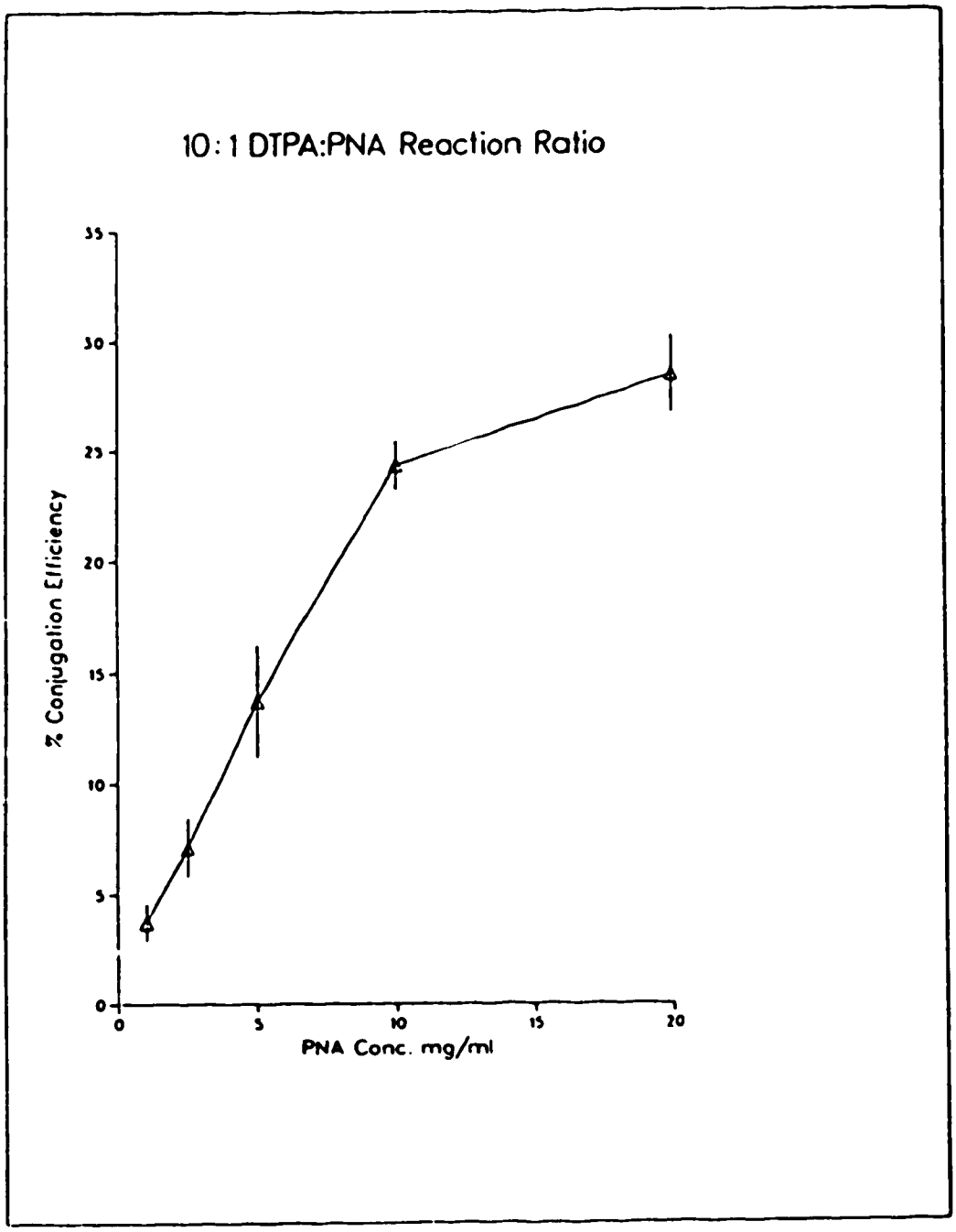
4.4.3 The Effect of Conjugation Levels on the Radiochelation of In-111

Another variable that strongly affected the number of DTPA chelates that can be attached to the PNA is the molar ratio of DTPA employed in the reaction. The number of

Table 18. The Effect of PNA Concentration on the DTPA-PNA Conjugation Reaction

PNA Concentration (0.2M PO_4 buffer, pH 8.5)	% Conjugation Efficiency ± S.D. (n=3)
1 mg/ml	3.72% ± 0.86
2.5 mg/ml	7.12% ± 1.34
5 mg/ml	13.70% ± 2.55
10 mg.ml	24.32 ± 1.12
20 mg/ml	28.46 ± 1.77

Figure 9. The Effect of PNA Concentration on the Conjugation Efficiency Between PNA and DTPA Dianhydride



chelates on the PNA molecule will in turn determine the labelling efficiency with In-111 citrate.

The number of DTPA chelate moieties attached per PNA molecule was determined by TLC utilizing two methods: the first method involves the addition of a tracer amount of In-111 citrate to the reaction mixture containing the DTPA coupled PNA and the free hydrolyzed DTPA. If the affinity of the two species to In-111 citrate was similiar, then the percent distribution of the In-111 tracer will reflect the relative abundance of the two species. Since the starting molar ratio of DTPA to PNA was carefully controlled, the average number of DTPA chelates attached to each of PNA molecule can be calculated by multiplying the percent conjugation efficiency with the molar ratio of DTPA to PNA used. The formation constant for amidic DTPA and free DTPA had been shown to be similar since the addition of stable indium carrier did not affect the percent efficiency calculated[267]. Another method that had been used to determine the conjugation level involved the prior removal of the free hydrolyzed DTPA from the conjugated PNA by dialysis, gel filtration or other methods. A tracer amount of In-111 was added to a standardized solution of stable indium citrate. A carefully calculated amount of the indium citrate standard (in excess of the DTPA-binding sites on the protein) was then allowed to react with the chelated protein. The amount of chelate on the protein can then be calculated by the binding capacity of the conjugated protein.

Both methods for the determination of the conjugation levels of the PNA were employed during this project, and the results obtained using the two methods are in close agreement with each other. Table 19 summarizes the calculations used for both methods and the results that were obtained.

With increasing molar ratios of DTPA to PNA, the percent conjugation efficiency decreases but the average number of DTPA residues attached per PNA molecule increases. The changes in conjugation and radiochelation efficiencies with increasing DTPA to PNA ratios can be seen in table 20. At very low DTPA to PNA ratio (1:1), a conjugation

Table 19. The Determination of the Average Number of DTPA Chelates on PNA by the Competitive Chelation Assay

Sample #	Method A[1]			Method B[2]		
	DTPA/PNA Ratio	% Chelation Yield	# DTPA Chelate/PNA	Amount DTPA-PNA Added	% Chelation Yield	# DTPA Chelate/PNA
1	3 : 1	44.25%	1.33	50 μg	11.36%	1.25
2	5 : 1	31.90%	1.60	50 μg	13.36%	1.47
3	10 : 1	27.80%	2.78	50 μg	19.18%	2.11
4	20 : 1	20.54%	4.11	50 μg	35.00%	3.85
5	50 : 1	13.82%	6.91	50 μg	56.45%	6.21

[1]10 μl of PNA conjugation mixture incubated with a tracer amount of no carrier added In-111 citrate.

[2]50 μg of purified DTPA-PNA incubated with 10 μl of 5 x 10^{-4}M solution of In-111 citrate.

Table 20. Effect of DTPA:PNA Conjugation Levels on In-111 Radiolabelling.

DTPA:PNA Reaction Ratio	% Conjugation	#DTPA/PNA Attached	% Radiolabelling Efficiency
1:1	9.00%	0.09	2.67%
2:1	46.50%	0.93	8.54%
3:1	41.67%	1.25	26.97%
5:1	32.80%	1.64	56.00%
10:1	28.40%	2.84	92.34%
20:1	22.30%	4.46	95.97%
50:1	15.64%	7.82	96.50%

efficiency of about 9% is observed. This may be attributed to the high rate of hydrolysis reaction which dominates at the low DTPA/PNA ratio. A certain amount of DTPA dianhydride may be required to overcome this problem and have the chance to react with the lectin molecule.

The radiochelation efficiencies of In-111 citrate with DTPA coupled PNA showed a direct relationship between the level of chelate attachment and the percent radiolabelling efficiencies. With a high level of chelate attachment, the radiolabelling of PNA easily exceeds 90%. Thus, it is possible to prepare DTPA coupled PNA as a kit preparation. With a suitable amount of DTPA attachment, the radiolabelling can essentially be completely in one simple step by adding the required amount of In-111 citrate.

However, on the other hand, as the level of chelation increases, this is usually accompanied by an undesirable decrease in the biological activity of the labelled protein[279-281]. The retention of the biological activities of the protein after radiolabelling is an important requirment during the radiolabelling step. Some heavily radiolabelled protein exhibited different *in vivo* behaviour such as a decrease in plasma half-live when it is compared to the undamaged protein[280]. When a certain average number of chelates were attached to the PNA, the Poisson distribution should be applied. So if an average target number of 3 chelates per molecule is chosen, then 5% of the PNA will fail to couple any chelate, 23% will posses three chelates and 13% will possess five chelates[280,281]. So a high percentage of the protein molecules will be heavily radiolabelled and they may account for a high percentage of the radioactive counts. It is these highly chelated protein molecules that are more prone to lose their native biological properties[280]. Therefore, it is usually necessary to compromise between the molar reaction ratio between DTPA anhydride and the retention of the T-antigen binding ability of the PNA to obtain a useful product. In this project, the aim was to obtain a biologically active product with a high labelling efficiency (>90%). From table 20, at a 10:1 molar ratio of DTPA to PNA, a relatively low level of chelate is attached but the radiochelation efficiency exceeds 90%. Thus, this reaction ratio was chosen for further evaulation of the retention of

biological activities and for subsequent biodistribution and radioimaging studies.

4.4.4 The Kinetics of the Radiochelation Reaction

The reaction kinetics of the radiochelation process were studied to determine the t of time required for complete chelation of the In-111 ions. The radiochelation yields a. .ious time intervals after the initiation of the radiochelation reaction were determined by TLC. Figure 10 represents the percent radiolabelling yield of a kit preparation of DTPA coupled PNA (reaction ratio 10:1 DTPA to PNA) when it was labelled with In-111 citrate. A plateau was reached after approximately 10 minutes, from then on, the radiochelation efficiency remained the same. On the basis of this experiment, the radiochelation reaction was always allowed to proceed for at least 15 minutes to ensure the completion of the chelating process.

The kinetics of the radiochelation reaction had also been shown to be affected by the concentration of the citrate ions in the reaction mixture. In-111 also forms a complex with the citrate ion and at high enough concentration , the citrate ion may compete with the DTPA for the In-111 ions and a longer reaction time may be required for complete chelation of the In-111 to the labelled protein[266].

4.5 Stability of the In-111 Labelled PNA

4.5.1 Stability on Storage at 25°C

The stability of the In-111 labelled PNA when stored at room temperature was tested by means of TLC analysis to determine the percentage of the radioactivity that was still protein bound at various time intervals. The results obtained are summarized in table 21. The purified In-111 labelled PNA retains over 98% of its radioactivity on the protein after 72 hours at room temperature.

Figure 10. The Radiochelation Efficiency Versus the Length of the Incubation Time

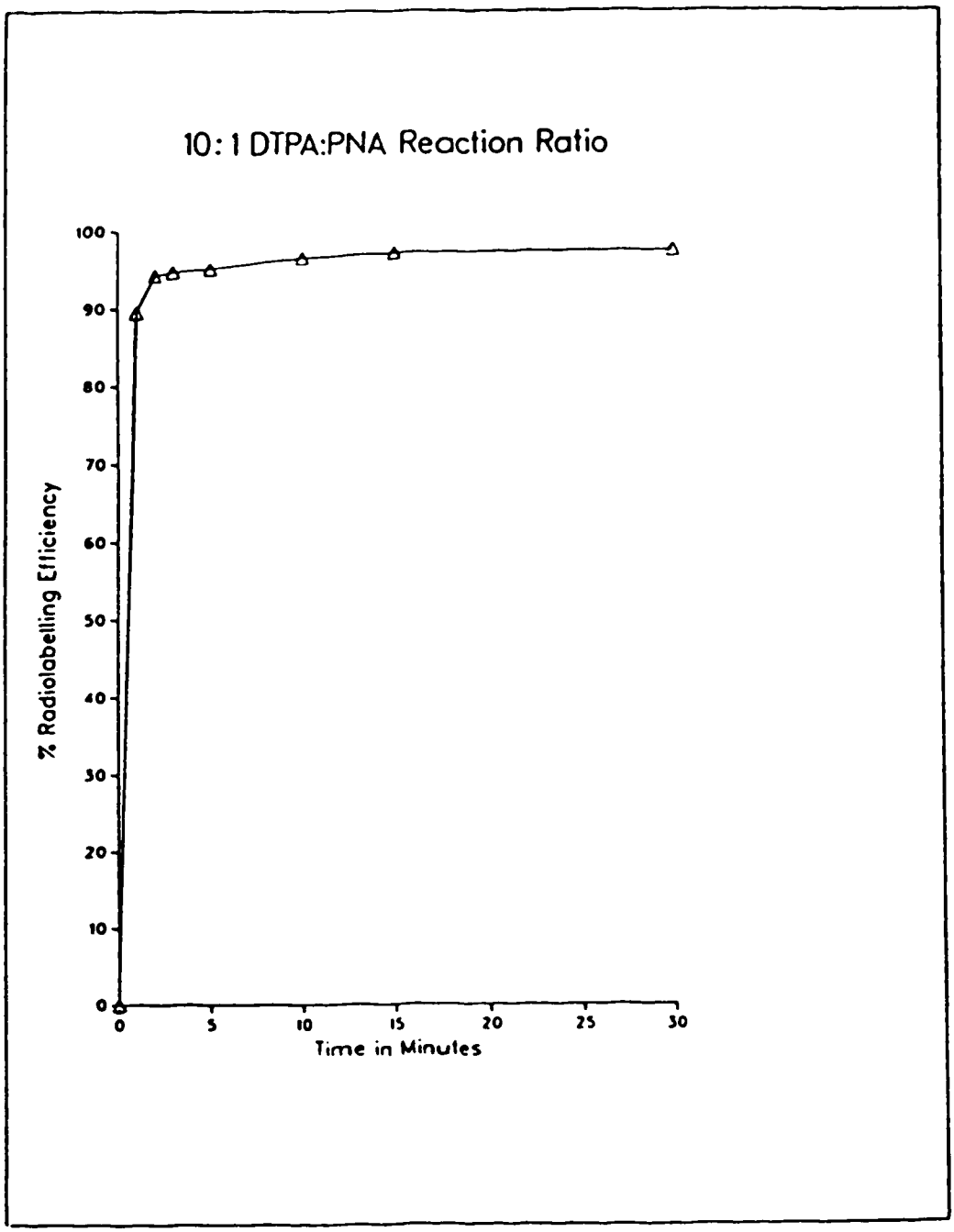


Table 21. The Stability of In-111 DTPA-PNA Stored at 25°C

Time	% Protein Bound Activity ± S.D. (n=3)	% Compared to Time 0
0 hr.	94.86 ± 0.58	100%
1 hr.	94.34 ± 1.52	99.45%
3 hr.	94.25 ± 1.29	99.36%
6 hr.	95.01 ± 0.56	100.16%
12 hr.	93.64 ± 0.56	98.71%
24 hr.	93.33 ± 0.94	98.39%
48 hr.	93.18 ± 0.88	98.23%
72 hr.	92.98 ± 1.73	98.02%

Such radiochemical stability of the In-111 labelled PNA will allow the use of the product 2 to 3 days after radiolabelling without significant loss of the radiolabel. However, due to the relatively short half-life of the radionuclide (T-1/2 = 67 hours), the labelled product is usually used within 24 hours to take advantage of the higher specific activity available.

4.5.2 Incubation With Chelating Agents

The In-111 ions attached to the DTPA portion of PNA must be resistant to transchelation to other chelating agents that may be present in the *in vivo* system. Incubation of the In-111 labelled DTPA-PNA complex with an excess of two of the commonly used chelating agents, DTPA and EDTA were used to assess the ability of the In-111 labelled PNA to resist transchelation. The stability of the chelated In-111 will depend on the relative stability constants and the relative molar ratios between the radiolabelled protein and the other available chelate sites. The stability constants of some of the commonly encountered In-111 chelate complexes are listed in table 22 [282,283]. In certain instances, although the stability constants of the chelate complex favors transchelation, the steady state of the reaction may take some time to reach and the labelled protein may have enough *in vivo* stability to allow it to localize to the region of interest[281,284,285].

The stability of In-111 labelled PNA when challenged with an excess of DTPA or EDTA ranging from a 1 to 10 molar excess of the free chelate are summarized in table 23. The molar ratio between the DTPA-PNA and the free EDTA or DTPA are calculated from the number of chelates that were being attached to the PNA molecule. Over 90% of the In-111 label was retained on the PNA molecule after 48 hours of incubation at room temperature. This provides evidence that the radiometal was effectively attached to the DTPA-PNA complex since any non-specifically associated radiometal would be removed by the challenging chelating agents.

Table 22. The Stability Constants of Indium (III) with Various Ligands[202,203].

Ligand	log K_1
OH^-	-2.11
SCN^-	+2.34
Cl^-	2.44
F^-	3.67
$CH_2O_2^-$(formate)	2.74
$C_2H_4O_2^-$	3.50
$C_4H_6O_5$ (tartrate)	4.48
$C_4H_4O_4^-$	5.00
$C_2H_2O_4^-$ (oxalate)	5.30
$C_6H_7O_7^-$ (citrate)	6.18
HEDTA (2-hydroxyl)ethylenediamine triacetic acid	17.20
EDTA	25.3
DTPA	28.4

Table 23. The Effect of Incubation with DTPA or EDTA on the Stability of In-111 Labelled PNA

Time	% Protein Bound Activity [1]				
	Control	DTPA to PNA ratio [2]		EDTA to PNA ratio [2]	
		1:1	10:1	1:1	10:1
0 hr.	94.86 ± 0.08	94.70 ± 0.62	94.56 ± 0.72	94.07 ± 0.38	93.86 ± 0.64
1 hr.	94.34 ± 1.52	94.08 ± 0.46	94.05 ± 0.89	94.10 ± 0.95	93.41 ± 0.72
3 hr.	94.25 ± 1.29	93.58 ± 0.87	93.88 ± 0.47	93.78 ± 0.87	92.36 ± 1.32
6 hr.	95.01 ± 0.56	93.60 ± 1.20	91.95 ± 1.21	93.64 ± 1.04	91.87 ± 0.56
12 hr.	93.64 ± 0.56	92.74 ± 1.18	91.03 ± 0.65	93.39 ± 1.52	91.64 ± 0.85
24 hr.	93.33 ± 0.94	91.08 ± 0.73	90.68 ± 0.83	92.78 ± 0.99	91.09 ± 1.03
48 hr.	93.18 ± 0.88	90.58 ± 0.96	89.73 ± 1.06	92.08 ± 1.05	90.30 ± 1.21

[1] Mean ± SD of 3 determinations.

[2] The DTPA or EDTA to PNA ratio is calculated according to the amount of chelate attached on the DTPA-PNA preparation.

Ward *et al*[286] studied the effect of various metal chelating agents on the biodistribution of an In-111 labelled antibody when they were injected into non-tumor bearing BALB/c mice. The chelating agents altered the biodistribution of In-111 citrate but did not affect the 48 hours tissue uptake of the In-111 labelled antibody. This study also suggested that the In-111 labelled proteins are quite resistant to transchelation to other chelating agents *in vivo* [286].

4.5.3 Incubation with Serum

The stability of the In-111 label in serum has a significant influence on the biodistribution of the radioisotope in the animal tumor model. Transchelation of the In-111 in serum will result in the formation of the transferrin-indium complex, which will in turn localize to the liver and bone[287,288]. If the amide bond between the DTPA and PNA is unstable, this will result in the release of In-111 DTPA into the circulation. It is well documented that In-111 DTPA will be promptly cleared through the kidneys[289]. In both cases, the loss of the In-111 label will adversely affect the uptake of the radiolabelled protein into the tumor, either due to a increase of background activity, or the rapid clearance of the radioisotope from the blood stream, thus diminishing the chance for tumor accumulation.

Thin layer chromatography cannot be used for the separation of the In-111 DTPA-PNA and the transchelation product In-111 transferrin since their Rf values are very similar. An indirect method was therefore used to measure the extent of transchelation. This procedure was based on the binding of In-111 DTPA-PNA to an immobilizied synthetic T antigen structure (T-Synsorb). While In-111 DTPA-PNA binds readily to the T-Synsorb, the product of In-111 transchelation does not possess affinity for this antigenic structure. Thus, as transchelation occurs, and the proportion of In-111 DTPA-PNA decreases, this will be reflected in a decrease in the T-Synsorb binding.

Table 24 lists the binding of In-111 DTPA-PNA to T-Synsorb (10 ng to 10 mg of T-Synsorb) after incubation with fresh rabbit serum at 37°C. The In-111 DTPA-PNA retained

Table 24. The Effect of Incubation with Serum on the T-Synsorb Binding Ability of In-111 DTPA-PNA

Time	% Binding to T-Synsorb[1]	
	Control[2] (n=3)	Incubated with Serum[3] (n=3)
0 hr.	46.58 ± 2.34	44.58 ± 0.84
6 hr.	44.[illegible] ± 2.10	38.80 ± 0.93
12 hr.	46.27 ± 3.50	37.06 ± 2.21
24 hr.	42.12 ± 0.86	35.57 ± 0.93
48 hr.	40.34 ± 1.68	31.31 ± 1.69

[1]Mean % ± S.D. 50 μl solution incubated with 10mg T-Synsorb for 8 hours.

[2]Incubated with 1 ml of PBS at 37°C.

[3]Incubated with 1 ml of serum at 37°C.

88% of its original T-Synsorb binding capacity after 48 hours of incubation . This reflects a gradual loss of In-111 from the labelled PNA in serum. Several investigators had measured the rate of transchelation of In-111 labelled protein to transferrin (the major metal ion chelating protein in serum)[271,284,285,290]. The bulk of the research was done on In-111 labelled albumin and In-111 labelled antibodies. The In-111 DTPA-antibody complex was incubated with serum at 37°C and the degree of transchelation to serum proteins was measured by a variety of methods. These included HPLC size exclusion chromatography[285,290], the separation of In-111 transferrin from the reaction mixture by affinity chromatography using an anti-transferrin column[284,290], or by the electrophoretic separation of the serum proteins on agarose gel followed by the measurement of radioactivity profile either by scanning with a radiation detecting strip scanner or by cutting the strip into small sections and counting the sections in an automatic well counter[290]. The *in vivo* stability of the radiolabelled compound in the animal or human can also be determined by collecting serum samples at various times post injection and subjecting the samples to the aforementioned assays[284]. In most cases, the rate of transchelation was found to be about 10% per day. Thus the values obtained from our studies are comparable to the values reported in the literature.

On the other hand, the incubation of In-111 DTPA with serum did not result in a significant loss of the In-111 label to the serum proteins[285]. Less than 5% of the radioactivity is lost after 10 days of incubation when it was analysed either by TLC or HPLC techniques[285]. Since one of the chelating arms of the DTPA molecule is utilized in the conjugation to the protein molecule only 4 ligands were available for the chelation of the radiometal. This slight change in the chelate structure may explain the difference seen on the stability of the radiometalchelation.

4.6 *In Vitro* Binding Studies

4.6.1 Binding to Control and Neuraminidase-Treated Red Blood Cells

The expression of T-antigen receptors on the surface of neuraminidase-treated red blood cells but not on corresponding normal red blood cells has been well documented[74-79]. Thus, neuraminidase-treated RBC can be used as a *in vitro* system for the measurement of the binding of radiolabelled PNA to the T-antigenic structure. The binding of radioiodinated PNA to neuraminidase-treated red blood cells had been studied by Zabel[101] and Eu[104]. In both studies, it was demonstrated that there was a significant amount of binding of I-125 PNA to neuraminidase-treated red blood cells but not to control red blood cells, suggesting that the radioiodinated PNA maintained its T-antigenic binding properties after radioiodination.

The amount of binding of I-125 PNA to neuraminidase-treated red blood cells of different blood group origins was not significantly different[104]. Thus, only blood group "O" blood was used in this experiment. The blood was kindly donated by the Canadian Red Cross Society blood transfusion service.

The binding of In-111 DTPA-PNA, I-125 PNA, In-111 citrate and In-111 DTPA to neuraminidase-treated and control red blood cells was measured to assess the degree of retention of T-antigen binding ability of the In-111 labelled PNA. In-111 citrate and In-111 DTPA were present in the study to assess the non-specific binding ability of the In-111 label and to ascertain that the binding of the In-111 labelled PNA is due to the interaction between the PNA and the T-antigenic structure and is not due to non-specific interaction between the In-111 label and the red blood cells.

The results of this study are summarized in table 25. Both the I-125 PNA and In-111 DTPA-PNA had high avidity for the T-antigen present on the exposed red blood cells while an insignificant amount of PNA binding were observed with the control red blood cells. This suggests that similar to radioiodinated PNA, the In-111 labelled PNA still retains its T-antigen affinity after the radiochelation procedure. In both cases, the presence of 0.05M D-galactose

Table 25. The Binding of In-111 DTPA-PNA and I-125 PNA to Control and Neuraminadase Treated Red Blood Cells.

	% Radioactivity Bound[1]			
	In-111 DTPA-PNA	I-125 PNA	In-111 Citrate	In-111 DTPA
Control RBC	2.20% ± 0.33	0.43% ± 0.06	0.26% ± 0.09	0.21% ± 0.02
Control RBC with 0.05M D-Galatose	1.43% ± 0.72	0.68% ± 0.41	0.31% ± 0.03	0.24% ± 0.05
N' RBC	29.25% ± 3.04	20.84% ± 6.89	0.33% ± 0.11	0.20% ± 0.03
N' RBC with 0.05M D-Galactose	1.22% ± 0.13	1.13% ± 0.42	0.25% ± 0.04	0.24% ± 0.07
Blank Control[2]	0.52% ± 0.26	0.60% ± 0.28	0.22% ± 0.02	0.20% ± 0.02
Blank Control with 0.05M D-Galactose	0.65% ± 0.08	0.55% ± 0.13	0.21% ± 0.03	0.21% ± 0.01

[1] Mean % bound ± S.D. of triplicate incubation of approximately 50KBq of In-111 DTPA-PNA, I-125 PNA, etc (500ng PNA) with 500 μl of 0.3% RBC or N'RBC.

[2] Mean % bound ± S.D. of triplicate determination. Blank control contains the radiolabelled species in 1ml of PBS only, no RBC or N'RBC is added.

significantly inhibited the binding of the radiolabelled PNA to the neuraminidase-treated RBC, thus providing evidence for the sugar specificity of the binding. Visual hemagglutination can also be observed upon incubation of the In-111 or I-125 labelled PNA with the neuraminidase-treated RBC, but not with the control RBC.

The binding of In-111 DTPA-PNA to the control RBC was low and the addition of D-galactose did not affect the binding significantly. This small amount of binding may be due to the non-specific interaction between the lectin and the RBC surface or due to the entrapment of a small amount of PNA solution between the packed RBC during the centrifugation procedure. There also appears to be a slightly higher level of binding for the In-111 DTPA-PNA than I-125 PNA to the control RBC. This probably reflects reflects a higher level of non-specific interaction with the control RBC as the binding is not affected by D-galactose inhibition.

In-111 citrate and In-111 DTPA do not show any significant level of binding to either the neuraminidase-treated or control RBC. Addition of 0.05M D-galactose did not have any influence on the binding. This is expected as neither In-111 citrate nor In-111 DTPA possess any affinity for the T-antigenic structure. This observation also suggests that the interaction of the In-111 DTPA-PNA and the neuraminidase-treated RBC is due to the lectin-T-antigen interaction.

4.6.2 Binding to Tumor Cell Lines

The binding of the radiolabelled PNA to T-antigen expressing and non-T-antigen expressing tumor cell lines was studied *in vitro*. The TA_3/Ha murine mammary adenocarcinoma tumor cell line is known to react with radioiodinated PNA as well as anti-T antibodies *in vitro* [104]. This tumor cell line sheds a high molecular weight glycoprotein epiglycanin which expresses the immunodeterminant carbohydrate structure of the T-antigen[99,105]. The TA_3/St subline is a non-epiglycanin secreting cell line which has also been shown to possess some PNA binding ability[67]. The RI lymphoma tumor cell line was used as a

T-antigen expressing animal tumor model and has been shown to express PNA binding sites *in vitro* [101]. The non-T expressing tumor cell line EL_4 is used as a control cell line.

The results of the *in vitro* tumor cell line binding of the radiolabelled PNA are shown in table 26. The binding of I-125 PNA to the T-antigen expressing tumor cell lines had been demonstrated by Eu[104] and Zabel *et al* [101]. From the results, both In-111 DTPA-PNA and I-125 PNA show a high binding avidity for the TA_3/Ha tumor cell line. The binding can be inhibited to a great extent by the addition of 0.05M D-galactose, thus showing the carbohydrate specificity of the binding. There are also significant amount of binding to other T-antigen expressing cell lines as compared to the control. D-Galactose is effective in inhibiting the binding of the radiolabelled PNA to all the T-antigen expressing cell lines studied[101,104].

The In-111 DTPA-PNA shows a higher degree of binding to the T-antigen expressing cell lines than the radioiodinated PNA. This may suggest that the In-111 labelled PNA may be more reactive towards the T-antigen than the radioiodinated lectin. The addition of iodine atoms into the protein molecule are known to affect the biological activity of the protein[277]. There has been some suggestion that the tyrosine residues of PNA are involved in the carbohydrate binding[291]. In constrast, free amino groups, which react in the DTPA conjugation reaction, did not appear to be involved in the PNA carbohydrate binding[291]. The radioiodination mainly involves the tyrosine moiety of PNA and this may have a negative effect on the bio-reactivity of the protein. Thus the In-111 labelled PNA may have retained its T-antigen binding ability somewhat better than the I-125 labelled PNA. It is also observed that there is a higher level of non specific binding associated with the In-111 labelled PNA. The binding to the EL_4 control cell line is 3.0% for the In-111 DTPA-PNA and only 0.49% for the I-125 PNA. This observation is consistent with the red blood cell binding study as a higher level of non-specific binding is also observed for the In-111 DTPA-PNA.

In-111 citrate and In-111 DTPA do not show any affinity for all the cell lines studied. This again confirmed the retention of the T-antigenic binding ability of the In-111 labelled

Table 26. The Binding of In-111 DTPA-PNA to T-antigen and Non-T antigen Expressing Tumor Cell Lines.

Tumor Cell Line	% Radioactivity Bound[1]			
	In-111 DTPA-PNA	I-125 PNA	In-111 Citrate	In-111 DTPA
TA_3/Ha	29.73% ± 3.47	19.94% ± 3.75	0.24% ± 0.04	0.30% ± 0.05
TA_3/Ha with 0.05M D-Galactose	2.45% ± 0.60	0.96% ± 0.24	0.31% ± 0.08	0.32% ± 0.17
TA_3/St	12.11% ± 1.48	5.96% ± 0.38	0.35% ± 0.10	0.35% ± 0.16
TA_3/St with 0.05M D-Galactose	1.92% ± 0.22	0.62% ± 0.14	0.37% ± 0.17	0.35% ± 0.06
RI	3.61% ± 0.16	0.67% ± 0.23	0.25 % ± 0.07	0.44% ± 0.12
RI with 0.05M D-Galactose	1.51% ± 0.07	0.56% ± 0.22	0.37% ± 0.22	0.41% ± 0.14
EL_4	3.00% ± 0.34	0.49% ± 0.04	0.29% ± 0.05	0.42% ± 0.23
EL_4 with 0.05M D-Galactose	1.58% ± 0.33	0.49% ± 0.73	0.25% ± 0.09	0.41% ± 0.28

Continued...

Blank Control[2]	0.52% ± 0.26	0.60% ± 0.28	0.22% ± 0.02	0.20% ± 0.02
Blank Control with 0.05M D-Galactose	0.65% ± 0.08	0.55% ± 0.13	0.21% ± 0.03	0.21% ± 0.01

[1] Mean % ± S.D. of triplicate incubation of approximately 2KBq of In-111 DTPA-PNA, I-125 PNA, etc. (20ng PNA) with 10^5 cells from each of the cell lines.

[2] Mean % ± S.D. of triplicate determination. Blank control contains only the radiolabelled species with 1ml of PBS. No tumor cells are added.

PNA.

4.6.3 Binding to Immobilized T-antigenic Structure

Another *in vitro* test for the T-antigenic binding ability of PNA can be performed using a synthetic T-discaccharide immobilized on inert support (T-Synsorb). The T-Synsorb express the α-configuration of the T-antigen dissacharide. PNA possess affinity for both the α and β form of T-antigen[209]. The binding of I-125 PNA to the asialo GM_1-synsorb which possess the β form of the T-antigen had been studied by Eu[104] and Boniface[209]. A high degree of binding to the asialo GM_1-synsorb was observed[104,209].

The binding of In-111 DTPA-PNA to the T-Synsorb is shown in table 27. A significant level of binding is observed for both the In-111 DTPA-PNA and the I-125 PNA. D-galactose inhibition of the binding is also observed. This further supports the claim that the In-111 labelling does not adversely affected the T-antigen affinity of the PNA. It also suggests that In-111 DTPA-PNA may be an potential tool in the scintigraphic detection of the T-antigen expressing tumors *in vivo*.

4.7 *In Vivo* Studies

4.7.1 The Biodistribution of I-125 PNA and In-111 DTPA-PNA

The biodistribution of I-125 PNA in mice bearing either the RI lymphoma or the TA_3/Ha mammary adenocarcinoma tumor models have been well documented in previous studies in these laboratories[101,104]. Shysh and Eu *et al* [236,237] have demonstrated the specificity of the radioiodinated PNA for the T-antigen expressing primary tumors as well as for the organ specific metastatic variants arising from the primary tumor using the TA_3/Ha murine tumor model. The rapid blood clearance of I-125 PNA coupled with the high tumor uptake in the TA_3/Ha tumor made this compound a promising agent in the scintigraphic detection of T-antigen expressing tumors[104]. The tumor to blood ratios of intravenously injected I-125

Table 27. The Binding of In-111 DTPA-PNA to T-Synsorb.

	% Radioactivity Bound[1]			
	In-111 DTPA-PNA	I-125 PNA	In-111 Citrate	In-111 DTPA
T-Synsorb	44.6% ± 2.58	40.8%± 3.67	0.83% ± 0.12	0.58% ± 0.09
T-Synsorb with 0.05M D-Galactose	15.3% ± 1.46	12.7% ± 0.84	0.92% ± 0.24	0.72% ± 0.13

[1] Mean % bound ± S.D. of 6 determinations. Approximately 3KBq of In-111 DTPA-PNA, I-125 PNA, In-111 Citrate, etc. (20ng PNA) were incubated with 10mg of T-synsorb for 8 hours.

PNA were 2.3:1, 7:1 and 55:1 at 6, 24 and 48 hours post injection. This allowed the clear scintigraphic delineration of the tumor mass without the need for background substraction techniques[104].

The specificity of the interaction between PNA and the TA_3/Ha tumor had also been evaluated by the simultaneous injection of a non-specific localizing agent labelled with a different radionuclide[104]. The localization index was calculated according to Pressman *et al* [292]. The localization index calculated for the uptake of I-125 PNA in the TA_3/Ha tumor was 1.19, 7.92 and 12.0 at 24, 48 and 72 hours post-injection[104].

However, as with many radioiodinated proteins, I-125 PNA also undergoes rapid *in vivo* deiodination. At 72 hours after intravenous injection in strain AJ mice, 74.5% of the total amount of radioactivity in the plasma samples were found to be free radioiodide[104]. The high level of circulating free radioiodide will be deposited into the kidneys, stomach and salivary glands, thus degrading the quality of the scan.

Another problem associated with the radiodinated PNA is the iodine label itself. I-125 can be used for scintigraphic studies in small rodents, but its low energy emissions preclude its use in larger animals or in humans. I-131, on the other hand, has been used routinely for radioscintigraphic studies in humans but its gamma energy is not particularly suited to the gamma camera[58,293]. I-123 presents with better energy conditions (159 KeV) but its short half-life and its high cost of production are practical limiting factors for use[58,293]. In-111 possess excellent physical properties for use in gamma ray scintigraphy and its 67 hour half-life is also very suitable for routine *in vivo* use.

Recent studies of tumor scintigraphy with In-111 radiolabelled monoclonal antibodies had revealed some advantages of the In-111 labelled antibodies over radioiodinated antibodies[280,284,293 - 296]. A major limitation of the radioiodinated antibodies is that upon break down in the tumor, the free radioiodide released will diffuse out of the tumor site into the general circulation. This results in a gradual loss of the radiolabel from the site of interest[261,284,294]. On the other hand, when the In-111 labelled antibody is metabolized in the

tumor, the In-111 released will be chelated to some intracellular components and be retained inside the tumor[289,294]. This will give rise to a longer half-life for the radiolabel in the tumor site and provide a better chance for imaging at a later time period.

The aim of this project therefore was to compare the *in vivo* biodistribution of PNA labelled with either I-125 or In-111. This will allow an assessment of the effect of the different radiolabelling procedures on the PNA molecule and a study to determine whether one method of labelling is superior to the other in terms of its final utility as an imaging agent for T-antigen expressing tumors.

The biodistribution of I-125 PNA and In-111 DTPA-PNA were performed as a dual label study in CAF_1/J mice bearing a solid subcutaneous TA_3/Ha tumor. Approximately 1 x 10^6 tumor cells were innoculated into the right flank of CAF_1/J mice. The tumor was allowed to grow for 10 to 14 days resulting in a well vascularized solid tumor of about 100 to 300 mg in size. I-125 PNA (74 KBq/0.5 μg) and In-111 DTPA-PNA (74 KBq/0.5 μg) were injected intravenously via the caudal vein into the mice and at time intervals of 1, 3, 6, 24, 48 and 72 hours post-injection of the radiotracer, a group of the mice were sacrificed , a blood sample withdrawn from the heart and the entire organs of interest were dissected out, weighted and placed into counting vials. The simultaneous injection of the two radiotracers gives rise to paired samples and will contribute to the decrease in intra-sample variations.

The tissues samples were first counted for radioactivity along with the injection standards in a Beckman 8000 well-type gamma counter using the dual isotope counting program which accounts for the spillover counts due to In-111 into the I-125 counting channel. The same samples were also recounted after 60 days for I-125 radioactivity since by that time the In-111 activity in the samples will be decayed away. The counts observed for I-125 were very similar to the counts obtained using the dual isotope counting program after decay correction. This confirmed that the dual isotope counting program was effectively correcting for the spillover of In-111 counts into the I-125 channel.

The results from the biodistribution studies are summarized in tables 28 and 29. From the tables, it can be seen that there is a rapid clearance of I-125 PNA from the body, whereas the clearance of In-111 DTPA-PNA is much slower. Whole body retention of the In-111 labelled PNA was 38.07%, 27.80% and 21.40% at 24, 48 and 72 hours post injection, while only 7.45%, 2.57% and 1.05% of the injected dose of I-125 PNA were left in the body at the same time period (figure 11) The major organs that are responsible for the retention of the indium label are the liver, spleen and kidneys.

The slow clearance of In-111 labelled protein compared to the radioinated protein have been observed by many researchers [284,293-295,297,298]. Most of these studies were done on In-111 labelled monoclonal antibody (MAb) used for detection of various types of malignancies. Otsuka *et al* [297,298], using a rat animal model, studied the biodistribution of monoclonal antibody HDP-1 labelled with either I-125 or In-111 (by cyclic DTPA anhydride or N-hydroxysuccinimide). The radioiodinated MAb or fragments clear rapidly from the non-target tissue so that by 24 hours post injection, low levels of radioactivity were observed in all tissue (except lungs, thyroid)[297]. With In-111 labelling of intact MAb, high uptake in liver is observed while the $F(ab)_2$ or Fab' fragments showed high uptake in the kidneys for the course of the study[297]. Thedreq *et al* [293] studied the tissue distribution of I-131 or In-111 labelled $F(ab')_2$ fragments of MAb 19.9 in nude mice bearing the human adenocarcinoma HT 29. The plasma clearance for the I-131 and In-111 were similar at 24 hr after infusion, but the liver, spleen and kidneys showed a high level of binding of the In-111 labelled $F(ab')_2$ [293]. High liver, spleen and kidney uptake of In-111 labelled antibody compared to radioiodinated MAb are observed in several other studies[284,293,297]. The reason for the long clearance half-live for In-111 is not clearly understood, and most researchers suggested that the difference between I-131 and In-111 labelled protein is the way the body handled the two labels[284,299]. Transchelation of the In-111 to iron binding protein in plasma directly or after metabolism of the antibody will produce In-111 transferrin and be deposited in the liver by ferritin or other iron binding proteins. Metabolism and/or dehalogenation of the

Table 28. Biodistribution of I-125 PNA in Mice Bearing TA,/Ha Tumor†

Organ		1 hour n=4	3 hours n=4	6 hours n=4	24 hours n=5	48 hours n=5	72 hours n=4
Blood	% dose/g	3.72 ± 1.01	2.78 ± 0.32	1.01 ± 0.27	0.16 ± 0.05	0.032 ± 0.01	0.02 ± 0.001
	% dose/organ	6.30 ± 1.63	4.53 ± 1.02	1.47 ± 0.06	0.31 ± 0.03	0.04 ± 0.006	0.03 ± 0.002
Liver	% dose/g	2.49 ± 0.72	1.85 ± 0.35	1.40 ± 0.35	0.61 ± 0.05	0.30 ± 0.02	0.24 ± 0.01
	% dose/organ	3.38 ± 1.23	2.35 ± 0.42	1.88 ± 0.25	0.79 ± 0.33	0.42 ± 0.12	0.31 ± 0.06
	organ:blood	0.95 ± 0.16	0.81 ± 0.14	0.93 ± 0.13	3.68 ± 1.23	11.15 ± 3.08	13.10 ± 2.08
Spleen	% dose/g	7.35 ± 1.17	3.50 ± 1.02	2.51 ± 0.18	0.34 ± 0.05	0.19 ± 0.02	0.08 ± 0.01
	% dose/organ	0.99 ± 0.11	1.66 ± 0.25	0.44 ± 0.02	0.12 ± 0.01	0.06 ± 0.007	0.02 ± 0.005
	organ:blood	2.01 ± 0.34	1.47 ± 0.28	2.77 ± 0.18	3.10 ± 0.62	5.94 ± 1.09	4.00 ± 1.28
Kidneys	% dose/g	75.09 ± 12.80	35.89 ± 7.64	20.13 ± 4.36	4.66 ± 1.22	1.40 ± 0.20	0.85 ± 0.14
	% dose/organ	33.67 ± 8.76	13.72 ± 0.76	6.82 ± 1.76	1.63 ± 0.35	0.47 ± 0.05	0.34 ± 0.04
	organ:blood	19.78 ± 2.65	14.99 ± 1.89	18.84 ± 2.23	34.81 ± 2.98	39.92 ± 5.76	41.89 ± 5.88
Tumor	% dose/g	2.98 ± 0.14	4.84 ± 0.67	4.98 ± 0.85	1.24 ± 0.07	1.02 ± 0.09	0.78 ± 0.05
	organ:blood	0.89 ± 0.12	2.40 ± 0.56	5.24 ± 1.10	7.01 ± 0.58	29.61 ± 3.07	38.71 ± 3.21
Lungs	% dose/g	4.03 ± 1.14	3.85 ± 1.28	2.51 ± 0.38	0.55 ± 0.07	0.14 ± 0.03	0.08 ± 0.005
	% dose/organ	0.58 ± 0.14	0.41 ± 0.12	0.39 ± 0.06	0.06 ± 0.01	0.02 ± 0.003	0.01 ± 0.002
	organ:blood	1.14 ± 0.28	1.42 ± 0.33	2.38 ± 0.34	3.38 ± 0.42	4.15 ± 1.18	4.10 ± 0.97
Salivary	% dose/g	5.10 ± 2.17	13.92 ± 4.32	18.40 ± 4.22	3.48 ± 1.07	1.58 ± 0.78	0.61 ± 0.13
	% dose/organ	1.10 ± 0.42	2.17 ± 0.76	2.95 ± 0.88	0.64 ± 0.21	0.21 ± 0.03	0.12 ± 0.03
	organ:blood	1.40 ± 0.44	5.01 ± 1.11	14.86 ± 1.62	24.15 ± 1.87	44.32 ± 4.98	33.20 ± 2.78

Continued...

Organ		1 hour n=4	3 hours n=4	6 hours n=4	24 hours n=5	48 hours n=5	72 hours n=4
Stomach	% dose/organ	6.03 ± 2.09	5.14 ± 1.54	6.42 ± 1.56	1.06 ± 0.38	0.54 ± 0.17	0.21 ± 0.03
	organ:blood	2.03 ± 0.35	3.66 ± 1.08	7.92 ± 2.08	5.15 ± 1.98	2.61 ± 0.94	2.36 ± 0.42
% injected dose remaining in carcass		31.62 ± 2.47	24.69 ± 3.54	13.40 ± 2.06	3.01 ± 0.42	0.81 ± 0.05	0.51 ± 0.07
% dose remaining in body except tumor		79.67 ± 5.64	54.67 ± 5.57	33.77 ± 4.69	7.45 ± 0.84	2.57 ± 0.17	1.05 ± 0.13

† Mean ± S.D. % of injected dose per gram of tissue and entire organ following i.v. injected of 74KBq (0.5 μg) I-125 PNA.

Table 29. Biodistribution of In-111 DTPA-PNA in Mice Bearing TA$_3$/Ha Tumor†

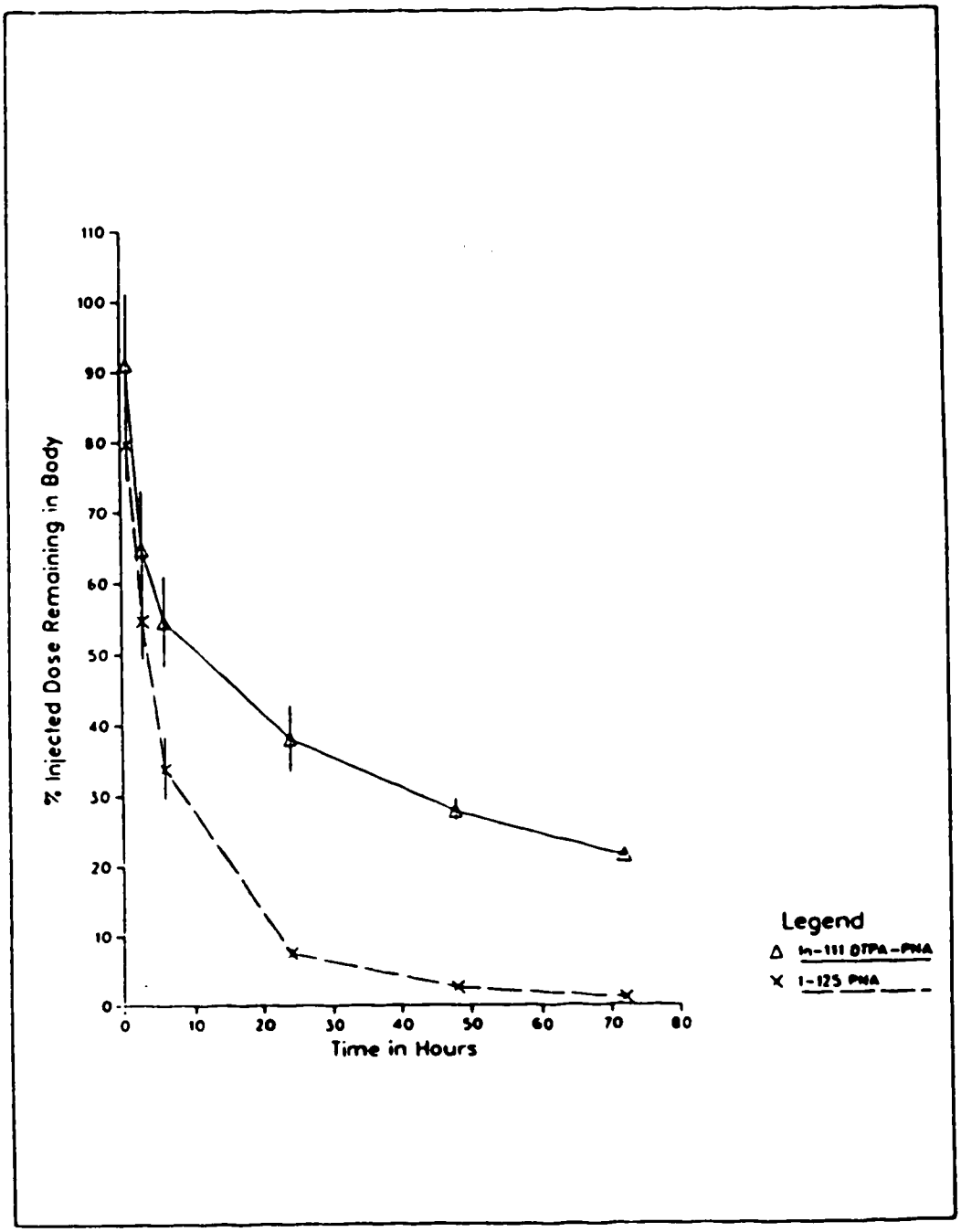
Organ		1 hour n=4	3 hours n=4	6 hours n=5	24 hours n=4	48 hours n=4	72 hours n=3
Blood	% dose/g	3.92 ± 1.09	1.31 ± 0.32	0.72 ± 0.06	0.34 ± 0.03	0.16 ± 0.01	0.09 ± 0.01
	% dose/organ	6.57 ± 1.72	2.38 ± 1.07	1.13 ± 0.11	0.48 ± 0.15	0.28 ± 0.09	0.13 ± 0.01
Liver	% dose/g	8.32 ± 0.48	7.38 ± 1.12	6.61 ± 0.18	5.79 ± 0.18	4.29 ± 0.29	3.06 ± 0.21
	% dose/organ	9.41 ± 0.75	8.92 ± 2.33	8.41 ± 0.47	7.68 ± 0.44	5.54 ± 0.39	4.73 ± 0.13
	organ:blood	2.12 ± 0.28	5.64 ± 0.86	9.20 ± 0.34	17.03 ± 2.17	26.91 ± 6.62	33.87 ± 1.56
Spleen	% dose/g	18.13 ± 3.72	6.52 ± 3.63	5.94 ± 1.72	4.88 ± 0.45	3.03 ± 0.71	2.31 ± 0.55
	% dose/organ	3.42 ± 0.35	3.57 ± 0.62	2.04 ± 0.24	2.10 ± 0.30	1.04 ± 0.09	0.74 ± 0.04
	organ:blood	5.12 ± 0.67	4.92 ± 1.25	5.31 ± 0.74	8.31 ± 0.85	17.47 ± 4.20	26.60 ± 10.54
Kidneys	% dose/g	83.98 ± 12.44	66.10 ± 19.16	61.05 ± 5.21	33.22 ± 2.72	25.47 ± 0.79	20.84 ± 2.24
	% dose/organ	37.62 ± 3.09	24.94 ± 5.58	24.21 ± 0.70	14.20 ± 1.78	11.14 ± 1.04	8.84 ± 0.99
	organ:blood	22.23 ± 4.38	45.15 ± 11.25	86.58 ± 7.90	117.19 ± 20.78	168.18 ± 43.89	221.56 ± 36.39
Tumor	% dose/g	5.62 ± 1.06	8.96 ± 2.28	6.98 ± 1.31	3.06 ± 0.91	3.02 ± 0.58	1.92 ± 0.30
	organ:blood	1.51 ± 0.38	5.17 ± 1.78	8.14 ± 1.92	11.50 ± 2.52	16.81 ± 2.98	21.89 ± 2.88
Lungs	% dose/g	4.13 ± 1.27	3.20 ± 1.08	1.87 ± 0.34	1.01 ± 0.25	0.80 ± 0.07	0.58 ± 0.03
	% dose/organ	0.66 ± 0.18	0.61 ± 0.22	0.29 ± 0.05	0.15 ± 0.02	0.10 ± 0.01	0.09 ± 0.01
	organ:blood	1.24 ± 0.35	2.23 ± 0.87	2.80 ± 1.01	3.90 ± 0.87	5.10 ± 1.24	6.18 ± 1.34
Salivary	% dose/g	2.12 ± 0.43	1.87 ± 0.18	1.92 ± 0.37	1.68 ± 0.26	1.86 ± 0.26	1.23 ± 0.03
	% dose/organ	0.45 ± 0.08	0.30 ± 0.10	0.36 ± 0.05	0.29 ± 0.03	0.34 ± 0.03	0.25 ± 0.04
	organ:blood	0.51 ± 0.06	1.34 ± 0.20	2.75 ± 0.38	4.90 ± 1.05	10.62 ± 1.76	13.66 ± 2.87

Continued...

Organ		1 hour n=4	3 hours n=4	6 hours n=5	24 hours n=4	48 hours n=4	72 hours n=3
Stomach	% dose/organ	0.28 ± 0.04	0.17 ± 0.02	0.16 ± 0.02	0.09 ± 0.01	0.07 ± 0.01	0.05 ± 0.01
	organ:blood	0.10 ± 0.02	0.31 ± 0.04	0.83 ± 0.20	1.15 ± 0.32	1.11 ± 0.24	1.01 ± 0.15
% injected dose remaining in carcass		32.67 ± 5.62	23.90 ± 2.76	18.00 ± 1.98	13.08 ± 2.08	9.08 ± 1.08	6.61 ± 0.88
% injected dose remaining in body except tumor		91.08 ± 10.17	64.70 ± 8.56	54.60 ± 6.47	38.09 ± 4.82	27.80 ± 1.90	21.40 ± 1.34

† Mean ± S.D. % of injected dose per gram of tissue and entire organ following i.v. injected of 74KBq(0.5 μg) In-111 DTPA-PNA.

Figure 11. Whole Body Retention of I-125 PNA and In-111 DTPA-PNA Following I.V. Injection in Mice

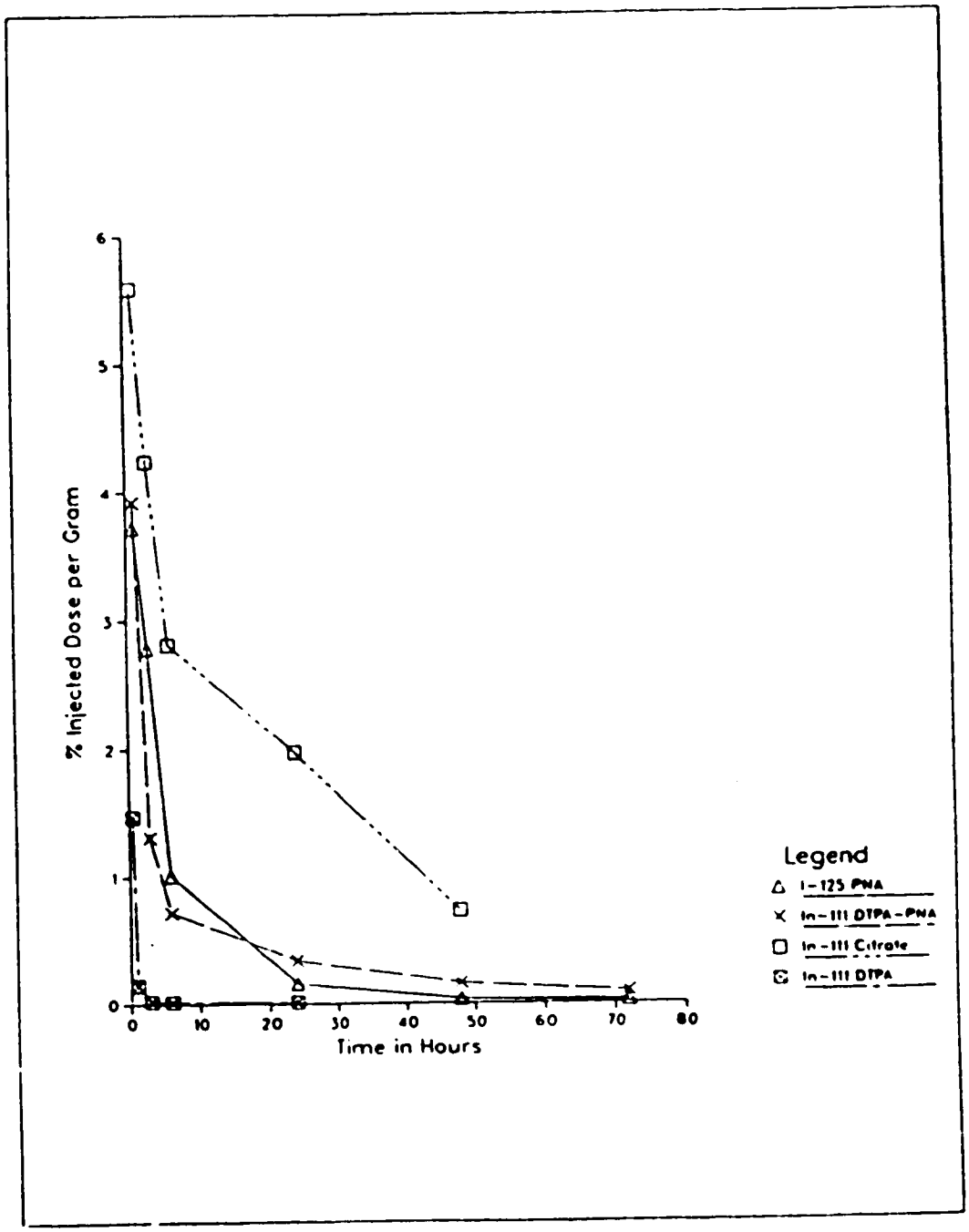


radioiodinated proteins will generate free radioiodide which can be cleared from the liver quite rapidly[299]. Studies with MAb internally labelled with Se-75 methionine also reveals a high level of antibody accumulation in the liver suggesting that the antibody may be actively metabolized in the liver[284].

The blood clearance of PNA is very rapid for both the I-125 and In-111 labelled PNA. Figure 12 shows the blood activity for both I-125 PNA and In-111 DTPA-PNA The clearance of In-111 DTPA-PNA appears to be biphasic with an initial rapid drop followed by a slower clearance rate after 24 hours. This biphasic clearance is also observed in MAb labelled with In-111 although the blood level of In-111 MAb is significantly higher than that with In-111 PNA[296,300]. At 48 hours post injection, 0.04% of the injected dose of I-125 PNA and 0.25% of the In-111 labelled PNA are left in the circulation (table 28 and 29). The higher blood levels of In-111 may be due to a slow transchelation of In-111 to transferrin which has a longer serum half-life. The rates of transchelation of In-111 to transferrin from various In-111 labelled antibodies have been estimated to be about 9-10% per day[284]. Hnatowich *et al*[284] suggested that since only labelled antibody in serum is exposed to transferrin, the degree of transcomplexation of In-111 MAb 19-9 was only about 1-2% of the administered activity due to the rapid blood clearance[284]. Since the blood clearance of In-111 DTPA-PNA is quite rapid, the effect of transchelation on the indium labelled lectin should be minimal.

The biodistribution of I-125 PNA in animal models has been well documented[101,104]. The data presented in table 28 and figures 11 to 15 are in general agreement with previous work. It was observed (table 28) that there was a rapid uptake of the I-125 PNA in the kidneys, the highest level was reached within the first hour and the activity slowly decreased with the excretion of the label from the kidneys. Biologically active PNA was also observed to be excreted in the urine although the high molecular weight of PNA (110,000) should exclude it from glomerular filtration[289]. Uptake in the tumor is significant and the radioiodinated lectin continued to concentrate in the tumor up to 6 hours post injection. This coupled with the rapid clearance from the circulation, gave rise to a high tumor to blood ratio of 15.6:1 at

Figure 12. The % Injected Dose Per Gram of Blood at Various Times Post I.V. Injection



24 hours and 38.7:1 at 72 hours. Uptake in other organs was low, about 3.38% of the injected dose was accumulated in the liver in an hour, but the activity was cleared rapidly from the liver. Uptake in the salivary and thyroid was observed some time after the injection, and this reflected the rapid *in vivo* deiodination of the protein. The uptake in the stomach may be attributed to the active secretion of free radioactive iodide, but it may also be due to specific interactions as some T-antigenic receptors are located in the stomach[104]. At 48 hours post injection, only the kidneys, tumor and the salivary glands retain significant amounts of the radioiodinated PNA activity.

The biodistribution of In-111 DTPA-PNA is quite different from the radioiodinated lectin as shown in table 29 and summarized in figures 13A-D. The major difference is the uptake in other non-target tissues, namely, the liver, kidney and the spleen. The liver accumulates 9.41% of the injected dose of In-111 DTPA-PNA within 1 hour and the activity is then cleared slowly with 4.73% of the injected dose left at the end of 72 hours. The kidney is by far the most significant organ in terms of the uptake of the radiotracer, accumulating 37.62% of the dose within 1 hour. This level is comparable to the I-125 PNA, but the In-111 DTPA-PNA remains in the kidney for a longer time. At 48 hours post injection, 11.14% of the injected dose of In-111 DTPA-PNA remains in the kidney compared to 0.47% with the I-125 PNA. No significant uptake of In-111 DTPA-PNA is apparent in the stomach, salivary glands or thyroid; this is expected since these organs are not involved in the metabolism of the In-111 label.

The localization of the two radiolabelled lectin compounds in the TA_3/Ha mammary tumors are presented in table 28 and 29 and summarized in figures 14 and 15. Maximum uptake is observed at about 6 hr for the I-125 PNA while the In-111 DTPA-PNA reaches the highest level in 3 hours post injection. The relatively high tumor uptake and rapid blood clearance of the radiolabelled lectins gives rise to tumor to blood ratios of 11.50, 10.81 and 21.89 at 24, 48 and 72 hours post injection for the In-111 DTPA-PNA and 7.01, 29.61 and 38.71 for I-125 PNA.

Figure 13A. The Biodistribution of I-125 PNA and In-111 DTPA-PNA in the TA_3/Ha Tumor Mouse Model 1 Hour Post I.V. Injection

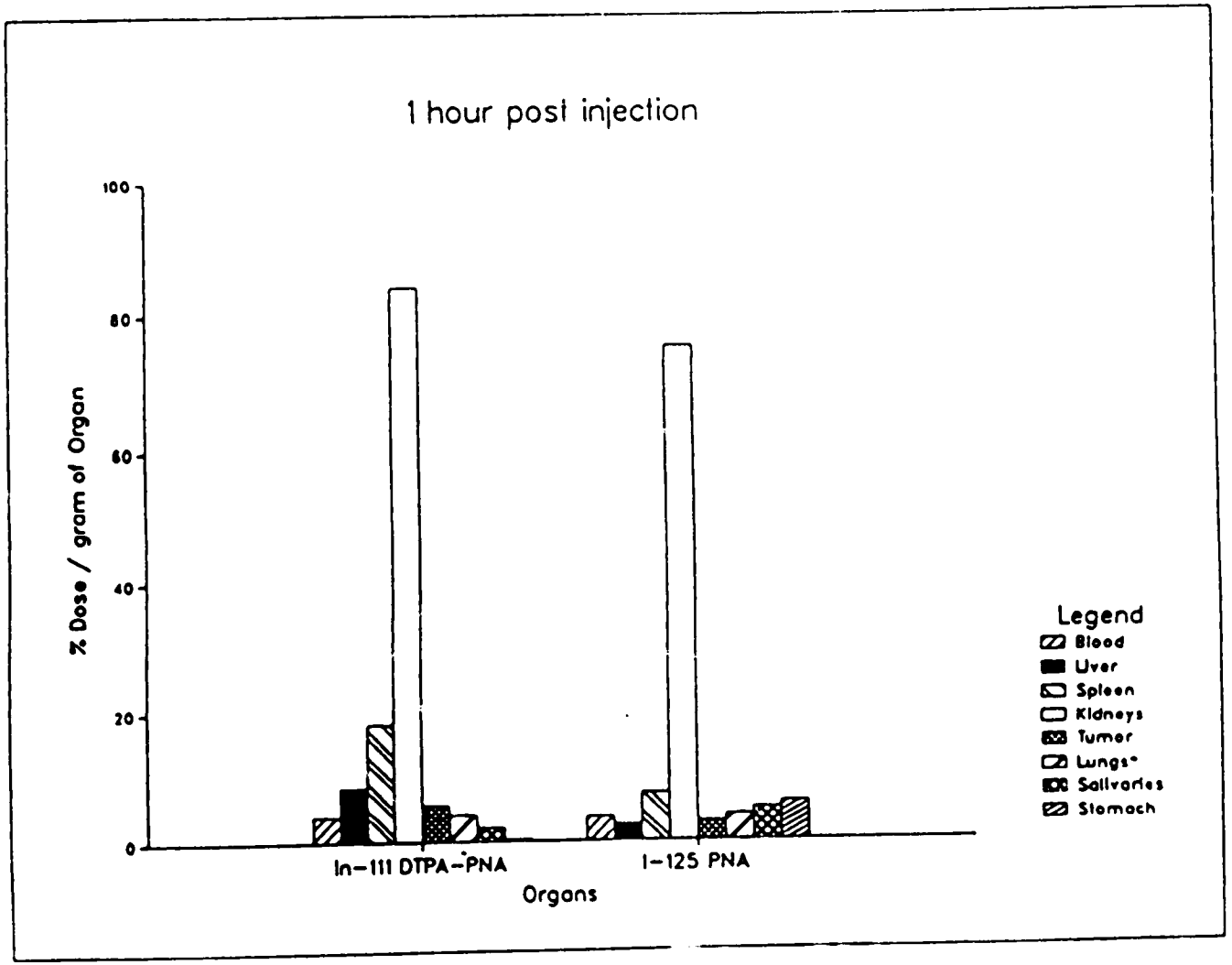


Figure 13B. The Biodistribution of I-125 PNA and In-111 DTPA-PNA in the TA_3/Ha Tumor Mouse Model 6 Hours Post I.V. Injection

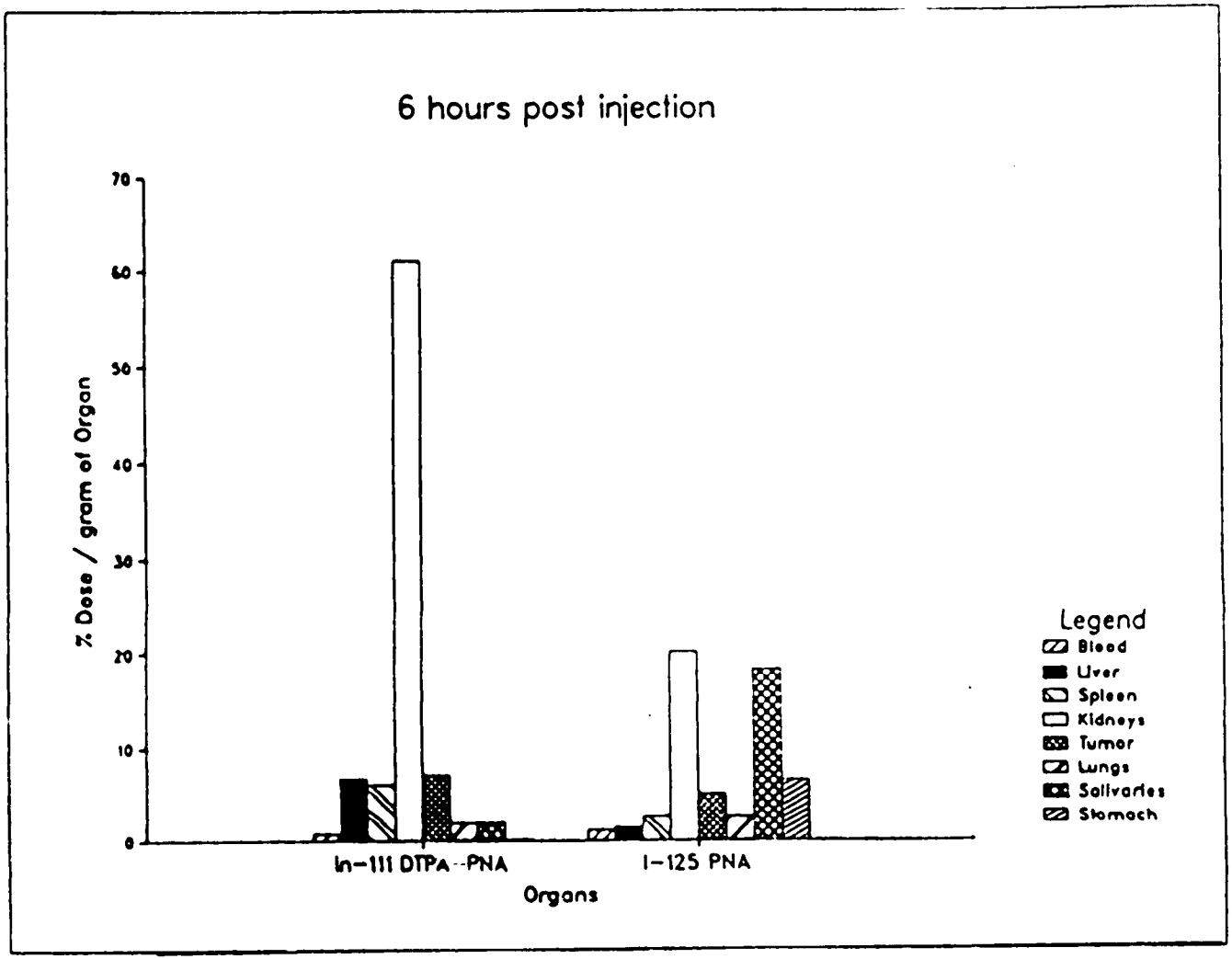


Figure 13C. The Biodistribution of I-125 PNA and In-111 DTPA-PNA in the TA_3/Ha Tumor Mouse Model 24 Hours Post I.V. Injection

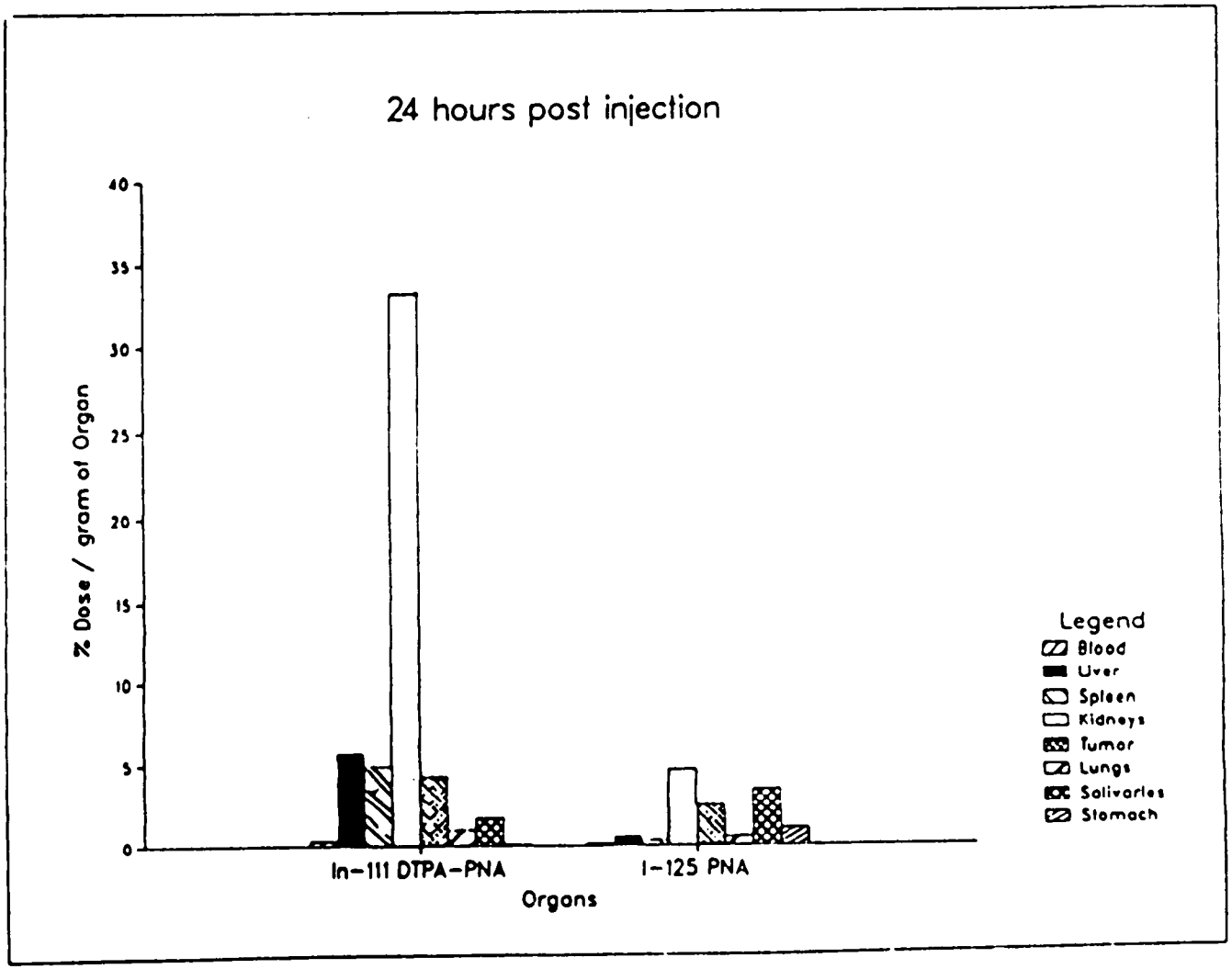


Figure 13D. The Biodistribution of I-125 PNA and In-111 DTPA-PNA in the TA,/Ha Tumor Mouse Model 72 Hours Post I.V. Injection

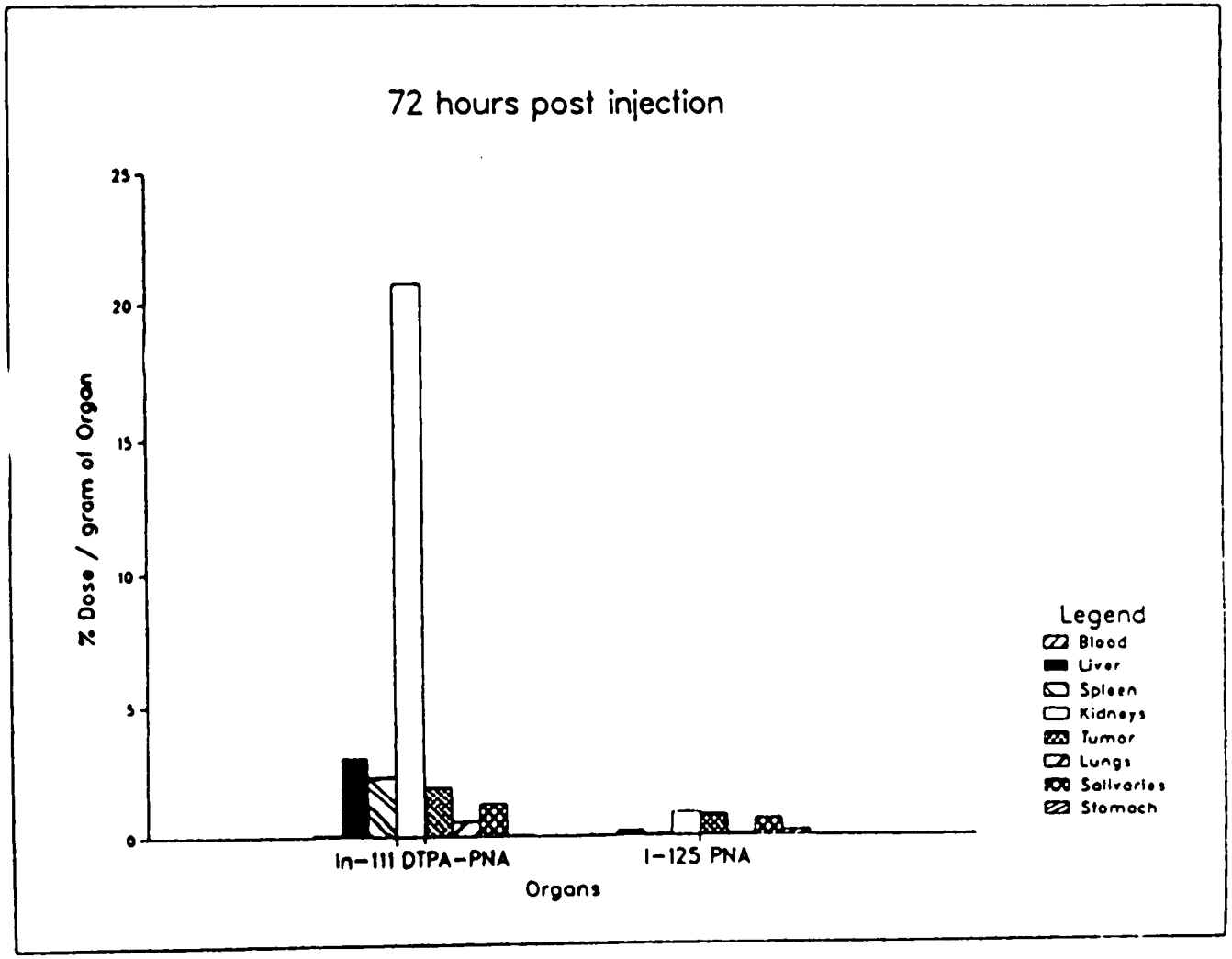


Figure 14. The % Injected Dose Per Gram of Tumor at Various Times Post I.V. Injection

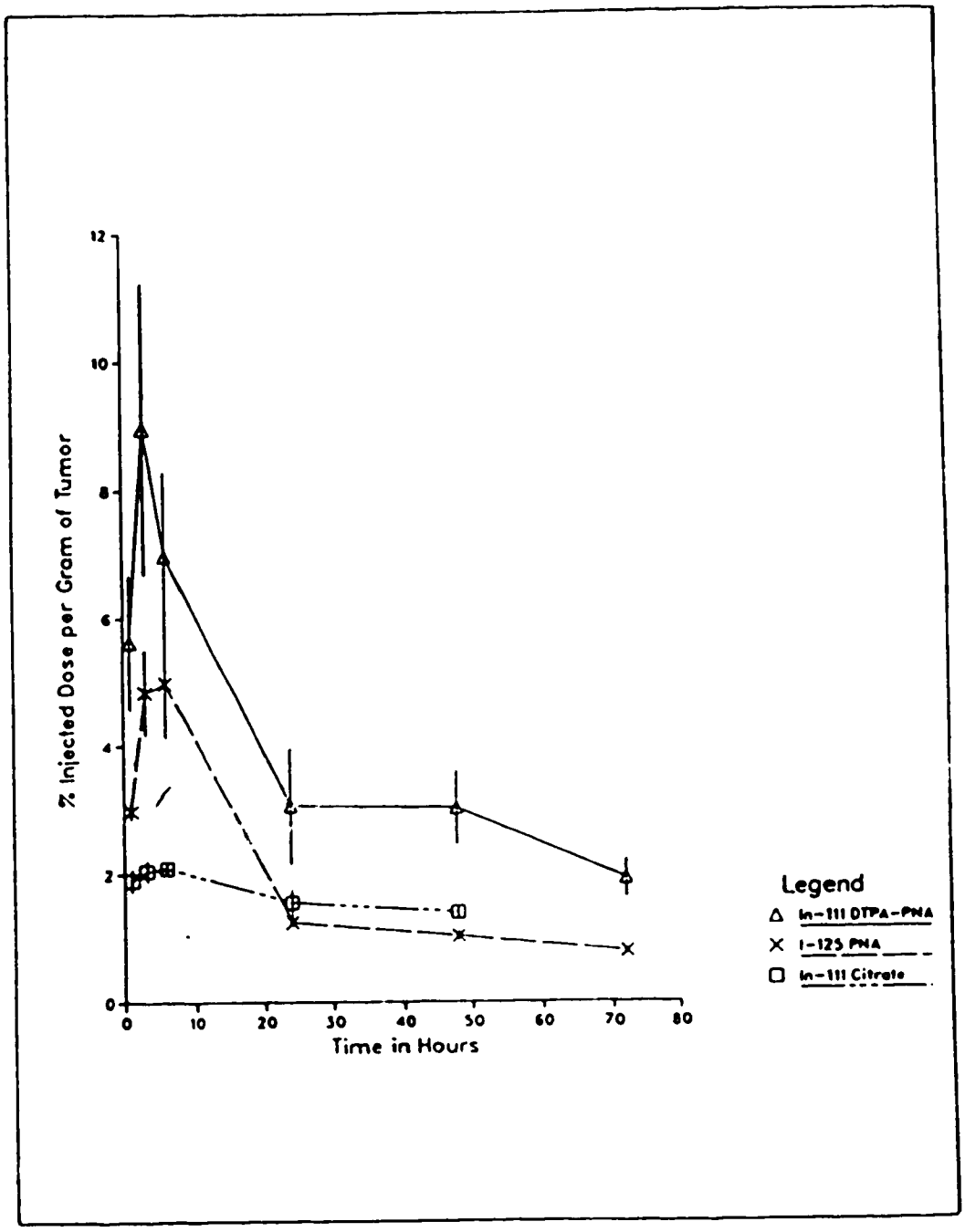
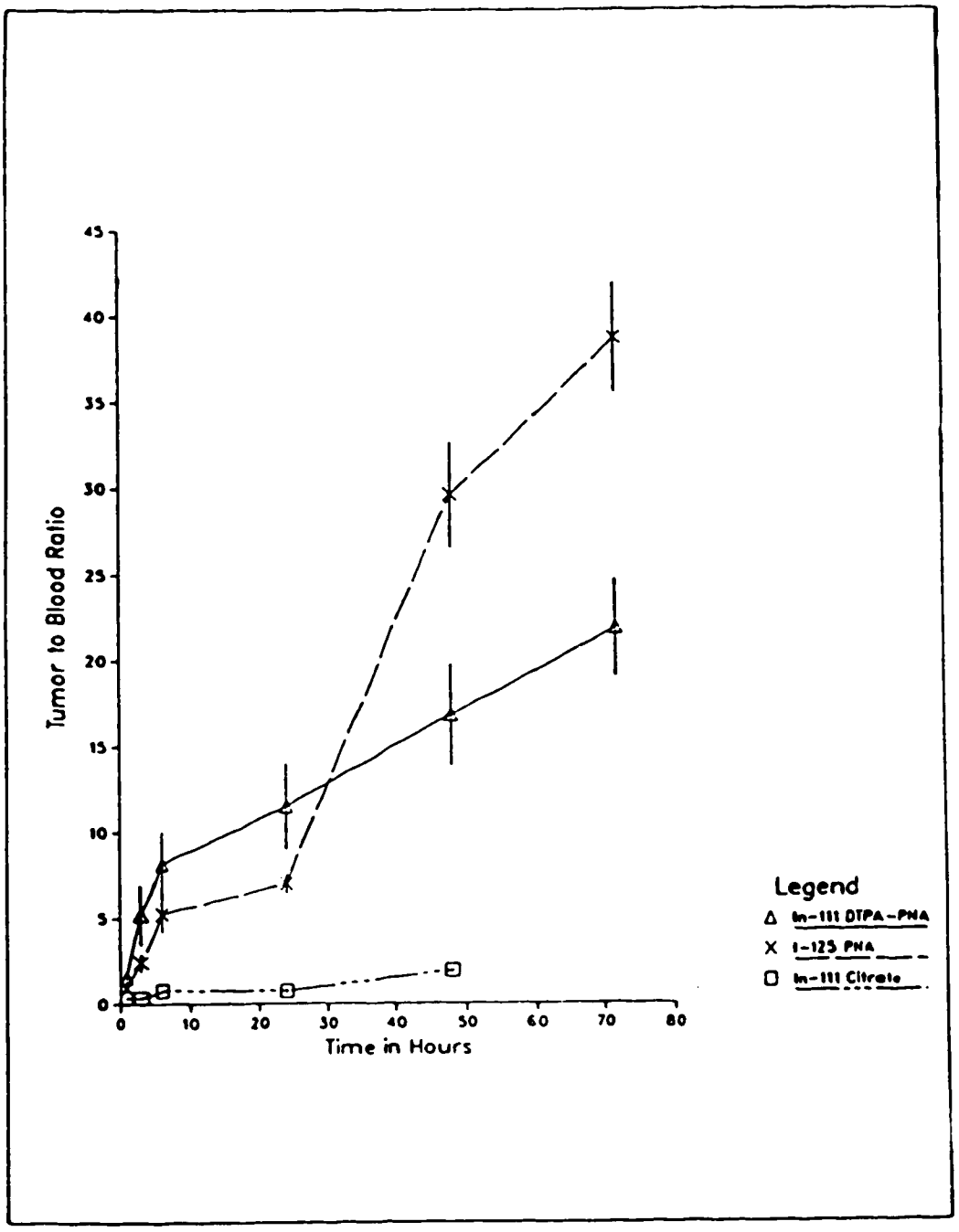


Figure 15. The Tumor to Blood Ratio at Various Times Post I.V. Injection

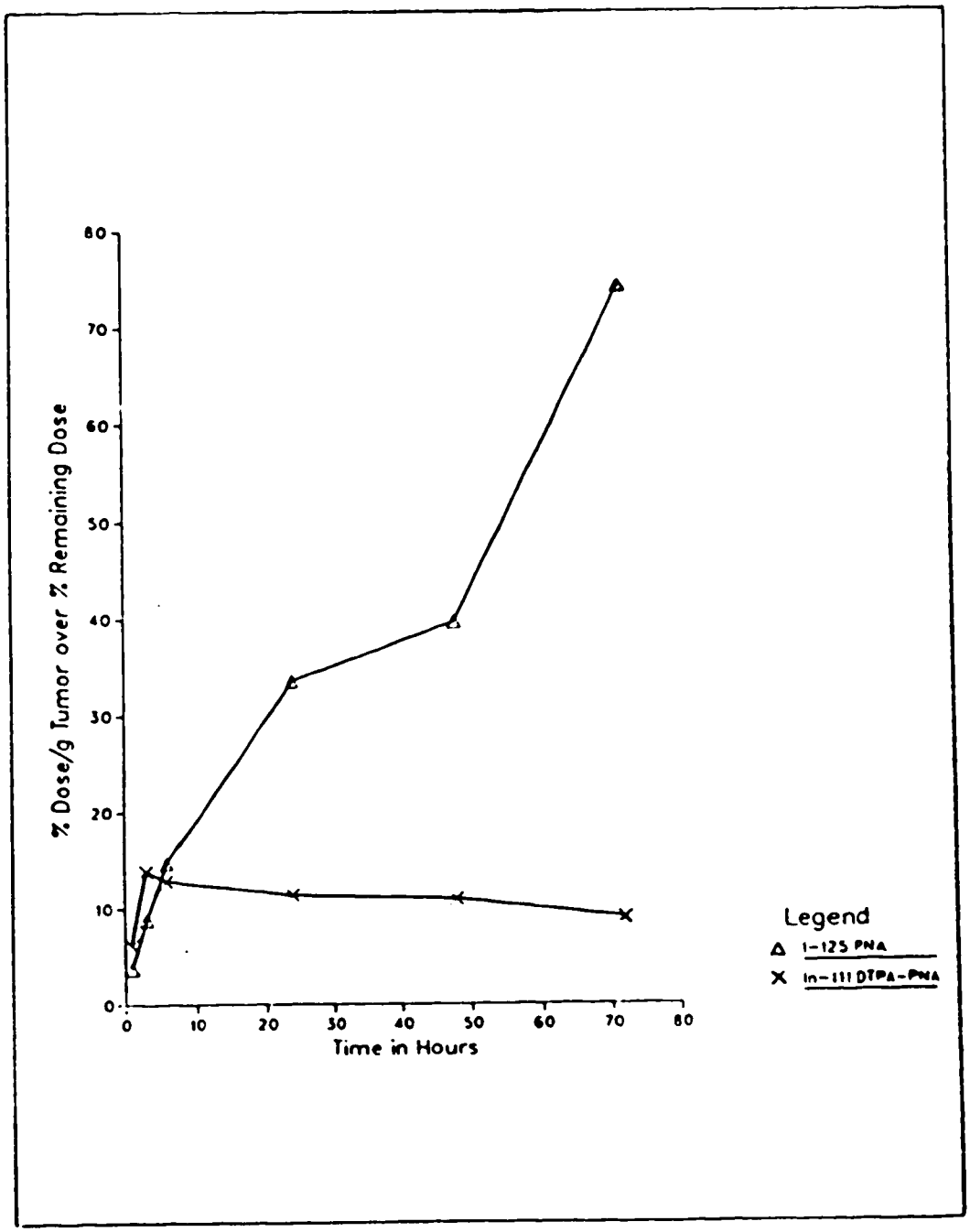


The uptake per gram of tumor is higher for the In-111 DTPA-PNA than for I-125 PNA, with about twice as much of the In-111 labelled lectin retained than the iodinated PNA at each time period (table 28 and 29). However, the total body clearance of the In-111 DTPA-PNA is slower thus resulting in a higher background activity. This can be seen clearly if the percent tumor uptake/gram is expressed as a percentage of the total dose remaining in the body at each time period (figure 16). The In-111 DTPA-PNA reaches a maximum of 13.85% at 6 hours and then the percent decreases slowly since the radiolabel is retained in the body. However, the value for I-125 PNA continues to increase as the I-125 label is excreted from the rest of the body rapidly.

Information obtained from studies with radiolabelled antibodies are in agreement of the results shown here. Several researchers have studied the biodistribution and immunoscintigraphy using In-111 and I-131 labelled antibodies in nude mice bearing human tumor xenographs[58,293,294]. The net tumor concentration of In-111 labelled antibody was found to be higher and retained longer than the corresponding radioiodinated antibodies , but the In-111 labelled antibody showed a lower tumor to liver and tumor to kidney ratio[293,294]. This may have a negative effect on immunoscintigraphy as the radiation dose to non-target tissues will increase and the constrast between tumor and non-target organs decreases. The high liver uptake may also prevent the detection of liver metastases[59] although in some cases the liver metastases showed up as cold spots in the liver[296].

Most of the researchers attribute the high tumor uptake of the In-111 labelled antibody to the stability of the indium label in constrast to the rapid *in vivo* dehalogenation of the radioiodinated antibodies [282,284,294,299,301]. Pimm *et al* [294] suggested two possible explanations for such increased tumor accumulation of In-111 labelled antibodies. The first explanation is that selective dehalogenation of the radioiodinated protein in the tumor occurs, releasing free radioiodide which is excreted from the tumor site. In-111 labelled proteins did not undergo dehalogenation and remained inside the tumor[294]. A second and more likely explanation is that both indium and iodine labelled proteins are being catabolized at the tumor

Figure 16. The Tumor Uptake of Radiolabelled PNA When Expressed as a Percentage of the % Dose Remaining in the Body



site, but while the liberated free radioiodide is excreted and removed, the free In-111 is retained in the tumor by metal chelating substances[294]. The exact nature of the retention of the In-111 label or the moiety that is responsible for its binding is not very well understood at present. However, it is usually observed that the whole body clearance of In-111 labelled antibody is much slower than its corresponding radioiodinated antibody, reflecting a general trend of retention of the In-111 label in the body[293,297]. Dessin *et al* [301] suggested that the fixation of In-111 in normal organs may be due to the binding to polyanionic mucopolysaccharides in connective tissues. Ward *et al* [216] studied the effect of circulating chelating agents and the pre-administration of carrier iron on the uptake of In-111 labeled antibody in tumor. The administration of chelating agents 24 hours post injection did not affect or remove the label from the tumor site[216]. It is also not possible to displace tumor bound label with carrier iron. This demonstrates that any free In-111 released from the antibody in the tumor was unavailable for complexation with external chelating agents and not displaced by iron[216].

Although the In-111 DTPA-PNA retains a higher percent injected dose in the tumor than the I-125 PNA, the high liver and kidney uptake of the In-111 radiotracer decreases the contrast between the target and non-target tissues. Figures 17 and 18 illustrate the tumor to liver and the tumor to kidney ratio for the two radiolabelled PNA complex. The tumor to liver or tumor to kidney ratio continues to rise with time for I-125 PNA as the radioactivity is cleared much faster in the liver or kidneys than in the tumor site. However, with In-111 DTPA-PNA, the highest ratio is reached in 3 hours post injection, but due to the retention of the indium in the liver and kidneys, the contrast between these organs and the tumor continues to decrease afterwards.

4.7.2 Retention of In-111 DTPA-PNA in Non-Target Organs

The accumulation of In-111 labelled protein in the kidney and liver is a commonly observed phenomenon but the reasons behind the localization are still not clearly understood

Figure 17. The Tumor to Liver Ratio for I-125 PNA and In-111 DTPA-PNA at Various Times Post I.V. Injection

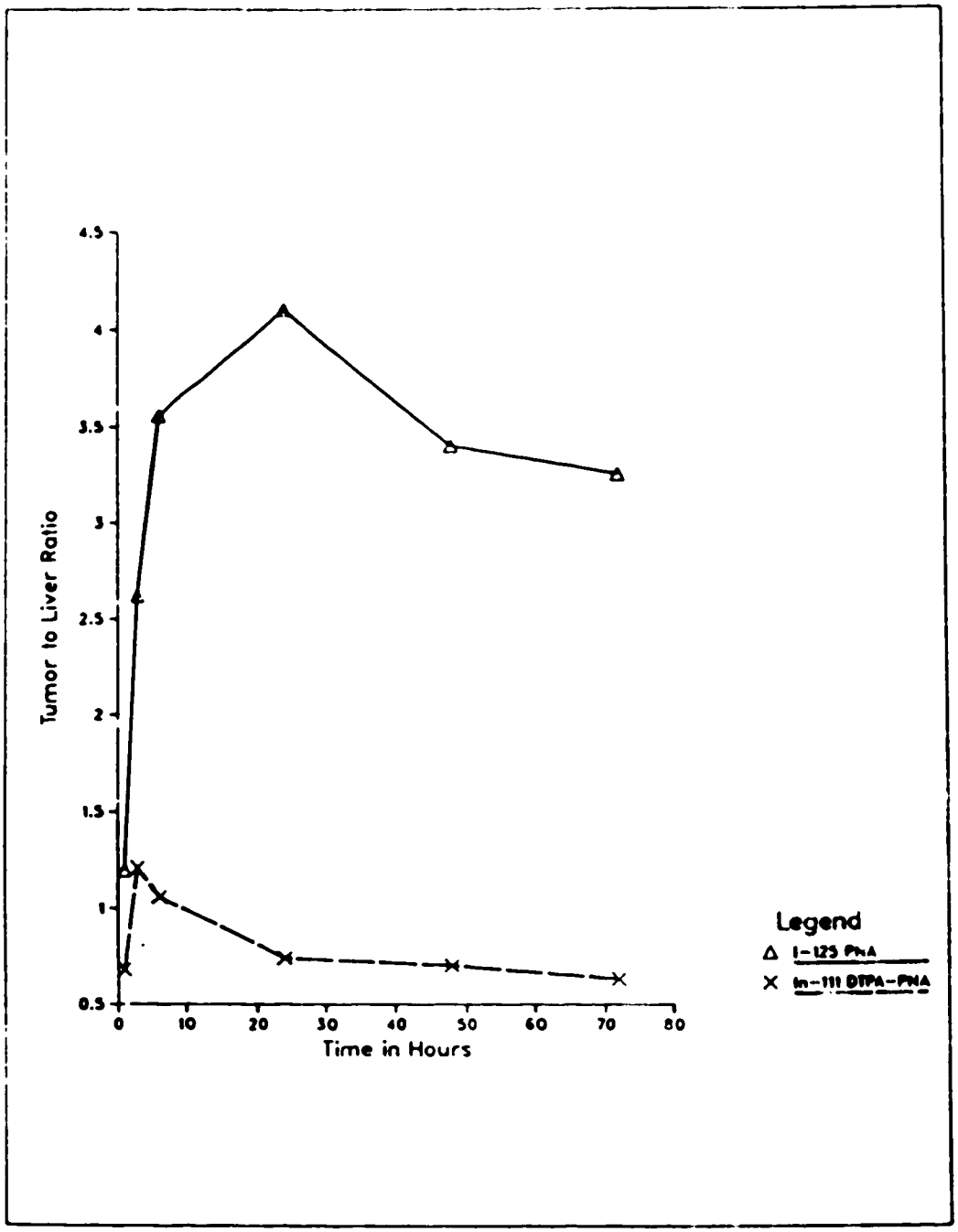
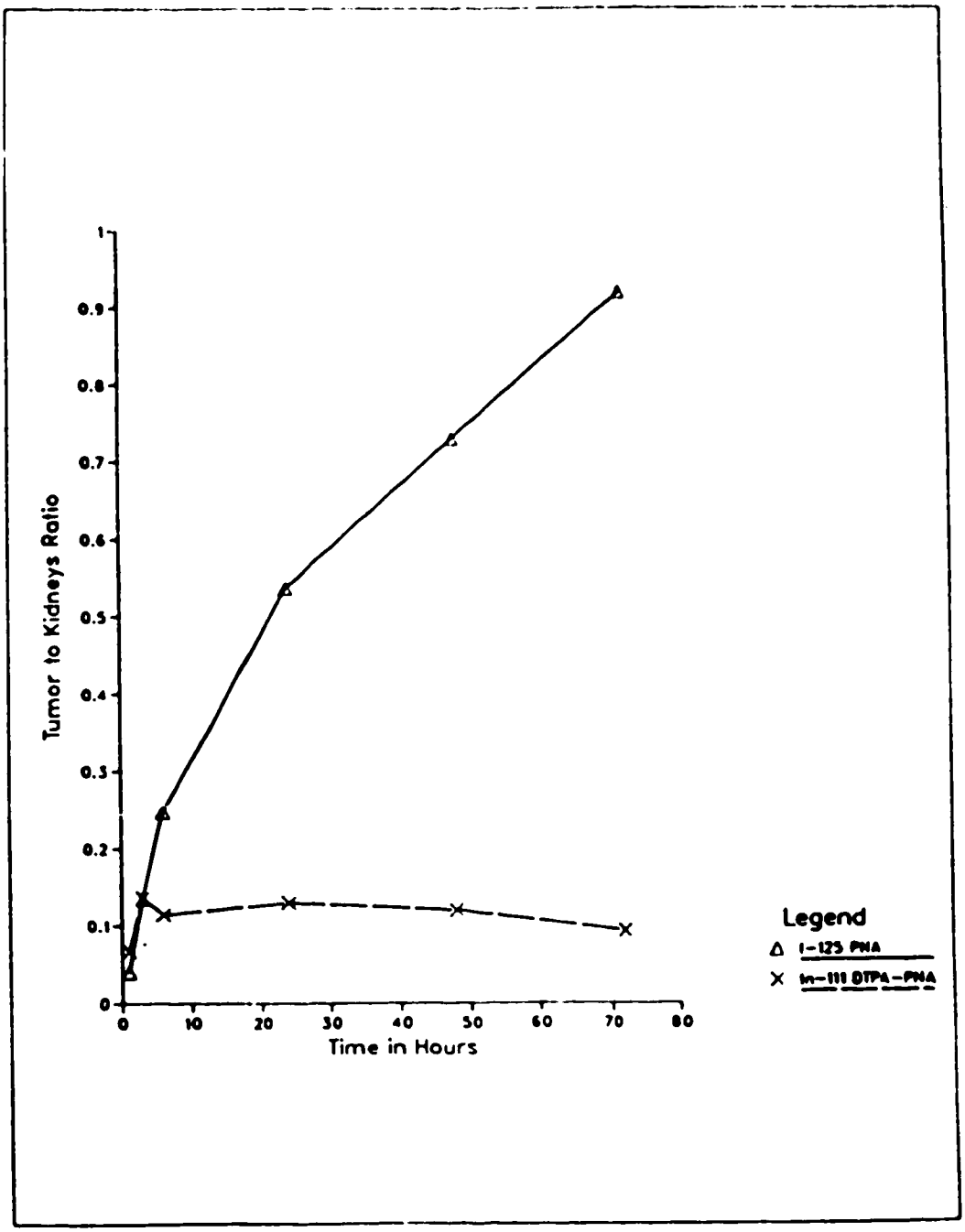


Figure 18. The Tumor to Kidney Ratio for I-125 PNA and In-111 DTPA-PNA at Various Times Post I.V. Injection



at the present [284,293,297,303,304]. The accumulation of the In-111 labelled protein in these organs not only decreases the target to non-target ratio of the radiotracer, it also increases the radiation absorbed doses in these organs and makes the detection of liver metastases difficult[59,301].

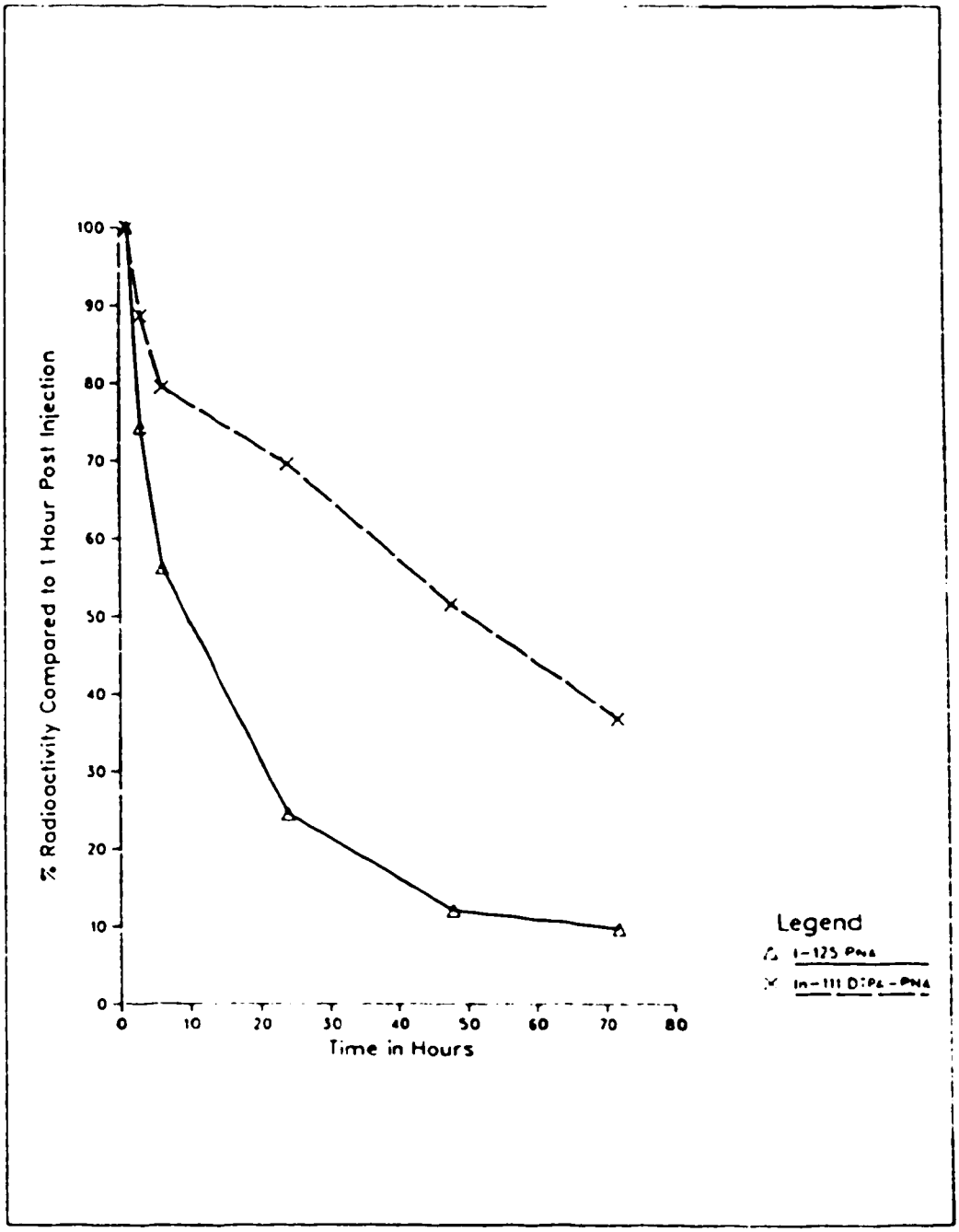
4.7.2.1 Liver and Spleen

A striking difference is observed when we compare the percent injected dose per gram of liver between I-125 PNA and In-111 DTPA-PNA. The clearance of In-111 DTPA-PNA from the liver is much slower than I-125 PNA . This comparison is easily seen if the amount of radioactivity in the liver is arbitrarily set to 100% at 1 hour post injection and if the percent dose per gram remaining in the liver at various time intervals is expressed relative to the level obtained at 1 hour post injection (figure 19).

The high liver uptake of In-111 labelled antibodies has been known for some time, however, the exact nature of the localization is still not very clear[284,293]. The time course of the accumulation of activity in the liver appears to reach its maximum level almost immediately and decrease slowly afterwards[284]. The difference in liver accumulation of the two differently labelled PNA may be due to the labelling process which changes the behaviour of the lectin or may be due to the sequestation of the In-111 label by the liver[301].

It may be tempting to assume that the uptake in liver is due to the *in vivo* instability of the In-111 label. Transchelation of the In-111 to transferrin can result in localization in the liver. However, the relatively slow rate of transchelation of In-111 to transferrin in serum cannot account for the rapid uptake of In-111 radioactivity in the liver[284]. The transchelation of In-111 to other proteins in serum cannot explain the decrease in liver In-111 level with an increased amount of carrier antibody injected which is observed in some cases [52,54,298,305]. Thus the In-111 transchelation in serum should not play a major role in the accumulation of the radiolabel in liver.

Figure 19. The Rate of Clearance of Radiolabelled PNA from the Liver



A dual label study with DTPA-PNA was performed to further investigate the effect of the labelling process on the PNA molecule. DTPA-PNA from a kit preparation was iodinated with I-131 using the Iodogen method. The I-131 DTPA-PNA was injected with the same amount of I-125 PNA as the control. The biodistribution of the two tracers were determined at 6 hr and 24 hr post injection. Table 30 summarizes the results of this experiment. The uptake of I-131 DTPA-PNA is found to be very similar to I-125 PNA with slightly higher uptake in the spleen for the I-131 DTPA-PNA. This may be due to the small amount of DTPA-PNA polymers that may be present in the injection dose and are being taken up by the spleen. The uptake in the liver is remarkably similar for the two radiotracers, suggesting that the addition of DTPA molecules to the PNA does not alter the way the body handles the radioiodinated PNA.

A similar experiment was performed by Khaw *et al* [306], in which he labelled a DTPA antibody complex first with I-125 and then with In-111. Prolonged retention of the In-111 was again seen in the spleen and liver. This seems to suggest that the difference in liver activity is due to the In-111 label and not due to the labelling process itself[306]. Studies with Se-75 antibody labelled internally express similar biodistribution patterns to the In-111 labelled antibody[301,307]. This suggested that the antibody is being taken up by the liver. During its retention in the liver, dehalogenation occured and the free radioiodine was cleared rapidly from the liver[301]. However, the In-111 label somehow remains associated with the liver after the catabolic mechanism[301,307]. Mathias and Welch [303] entrapped C^{14}-labelled DTPA complexed with stable indium and a trace of In-111 in liposomes. The liposomes were administered in a rat model. The fate of these two radiotracers were found to be different as In-111 was retained in the liver while the C^{14} activity was cleared largely through the kidneys[303].

The exact species that is reponsible for the retention of In-111 in the liver is still not very clear at the present. Iron binding proteins in the liver are thought to play a important role[301]. Shocket *et al* [308] studied the metabolism of In-111 labelled antibodies in

Table 30. Biodistribution of I-125 PNA and I-131 DTPA-PNA Dual Label Study in CAF_1/J Mice

Organ		I-131 DTPA-PNA		I-125 PNA	
		6 hours n=3	24 hours n=3	6 hours n=3	24 hours n=3
Blood	% dose/organ	3.09 ± 0.83	0.19 ± 0.03	2.65 ± 0.86	0.16 ± 0.03
	% dose/g	2.56 ± 0.55	0.14 ± 0.03	2.21 ± 0.67	0.12 ± 0.03
Salivary	organ:blood	7.54 ± 2.21	10.59 ± 1.35	9.06 ± 1.57	12.36 ± 1.92
	% dose/organ	2.84 ± 0.54	0.25 ± 0.01	2.96 ± 0.54	0.24 ± 0.01
	% dose/g	18.54 ± 3.26	1.50 ± 0.23	19.32 ± 3.23	1.44 ± 0.21
Spleen	organ:blood	4.11 ± 1.54	12.30 ± 2.00	2.35 ± 0.73	6.11 ± 1.14
	% dose/organ	0.93 ± 0.06	0.18 ± 0.01	0.46 ± 0.05	0.07 ± 0.01
	% dose/g	9.94 ± 1.43	1.74 ± 0.37	4.90 ± 0.91	0.72 ± 0.20
Stomach	organ:blood	6.81 ± 2.36	3.62 ± 0.56	7.91 ± 1.01	3.82 ± 0.62
	% dose/organ	4.05 ± 1.98	0.26 ± 0.04	4.14 ± 1.91	0.23 ± 0.04
	% dose/g	17.40 ± 7.33	0.53 ± 0.19	17.82 ± 6.99	0.47 ± 0.19
Liver	organ:blood	0.64 ± 0.13	2.21 ± 0.04	0.65 ± 0.07	2.63 ± 0.13
	% dose/organ	1.77 ± 0.35	0.41 ± 0.05	1.57 ± 0.35	0.40 ± 0.04
	% dose/g	1.59 ± 0.28	0.32 ± 0.08	1.41 ± 0.29	0.31 ± 0.07
Kidneys	organ:blood	16.76 ± 4.33	58.81 ± 4.21	18.90 ± 4.06	51.98 ± 3.17
	% dose/organ	14.18 ± 1.18	3.11 ± 0.45	13.67 ± 0.89	2.27 ± 0.39
	% dose/g	41.25 ± 4.71	8.39 ± 1.61	39.78 ± 4.18	6.14 ± 1.33

Continued...

Organ		I-131 DTPA-PNA		I-125 PNA	
		6 hours n=3	24 hours n=3	6 hours n=3	24 hours n=3
Lungs	organ:blood	2.93 ± 2.56	7.03 ± 4.55	3.08 ± 2.05	6.26 ± 4.59
	% dose/organ	0.88 ± 0.52	0.14 ± 0.11	0.87 ± 0.46	0.10 ± 0.08
	% dose/g	6.61 ± 4.20	1.03 ± 0.81	6.52 ± 3.72	0.75 ± 0.61
Muscle	organ:blood	0.38 ± 0.13	1.28 ± 0.22	0.32 ± 0.07	0.73 ± 0.35
	% dose/g	0.98 ± 0.45	0.18 ± 0.06	0.68 ± 0.09	0.09 ± 0.06
Bone	organ:blood	0.57 ± 0.14	1.39 ± 0.15	0.57 ± 0.06	0.75 ± 0.14
	% dose/g	1.44 ± 0.40	0.20 ± 0.03	1.24 ± 0.36	0.09 ± 0.02

the liver of guinea pigs. The homogenized liver extract was studied by size-exclusion HPLC. The radioactivity was found to be contained in 3 major peaks corresponding to ferritin, intact antibody and a low molecular weight fraction. The bulk of the radioactivity was associated with the unidentified low molecular weight fraction[308]. Although the exact nature of this interaction is not known, the In-111 which was fixed in the liver was very stable and resistant to transchelation. Infusion of various chelating agents 24 hours post injection of the In-111 antibody did not remove the radioactivity from the liver[286].

The concept of generalized clearing of foreign proteins by the reticuloendothelial system (RES) prompted some researchers to try to block the liver uptake by affecting the RES function[301]. Sands and Jones[301] pretreated the mice with dextran sulfate to induce RES blockade. The nature of the blockade may be a direct action on the Kupffer cells or due to the removal of a factor necessary for RES function. The RES blockade did not change the localization of the antibody in the liver suggesting that the liver uptake may be due to antibody binding to hepatocytes[301].

Receptor interactions between the radiolabelled proteins and the liver are also thought to play a role in the binding of In-111 to the liver. Liver contains non-specific Fc portion receptors for the antibodies which may be responsible for the high liver uptake of radiolabelled antibodies[296,299,305]. Removing the Fc portion of the heavy chain will sometimes reduce the localization of the antibodies in the liver and spleen and this is thought to be due to the decrease in non-specific interactions with the Fc receptors in the liver[299]. Recently, there are an increasing number of reports which show better tumor visualization and reduced liver uptake with increasing amount of antibody dose. This has been shown in both animal and humans studies[46,52,54,298,305]. Otsuka and Welch [298] studied the dose effect of In-111 HDP-1 antibody on the lung and liver uptake in a rat animal model. The dose of the antibody injected ranged from 2.5 to 500 μg per rat. The percent dose per gram of the target(lungs) to percent dose per gram of liver changed

from 0.26% at 2.5 μg dose to 2.68% with the 500 μg dose[298]. Murray *et al* [47] utilized In-111 labelled antibody 96.5 raised against the p97 antigen for imaging of malignant melanoma in human. The antibody dose ranged from 0.5 mg up to 20 mg. The percentage of metastases imaged ranged from 0% with the 0.5 mg dose up to 81% with the 20 mg dose labelled with 5.0 mCi of In-111[47].

The improvement of the image quality with larger MAb dose was attributed to the saturation of non-target organ uptake with "cold" antibody[305]. This effectively increases the total antibody concentration available for the slower uptake of In-111 labelled MAb by the tumor. The uptake of In-111 labelled MAb by the liver seems to be associated with a saturable mechanism. The uptake or metabolic process which is being saturated has not been determined yet[298].

Alvarez *et al* [309] has demonstrated that site-specifically modified In-111 labelled antibodies give low liver backgrounds and improved radioimmunoscintigraphy. The antibodies were modified at their carbohydrate moiety by oxidation of the oligosaccharides followed by the conjugation with the bifunctional chelating agent aminoaniline diethylenetriaminepentaacetic acid (AADTPA)[309]. The biodistribution data did not show substantial uptake or localization to non-target tissues and good uptake in the tumor. The addition of about 5 chelates per antibody did not affect the immunoreactivity of the antibody since the modified carbohydrate are present at the Fc regions of the antibody. The In-111 labelled antibody was quite stable *in vivo* and resisted transchelation to transferrin or other metal binding proteins[309]. The results seem to suggest that carbohydrates in the Fc portions of the antibodies may have some effects on the biodistribution of the In-111 labelled antibody. However, since PNA does not possess a Fc region or covalently linked carbohydrates[175], the mechanisms of its binding in the liver may be different from that seen with the In-111 labelled antibodies.

Another possible explanation for the high liver localization of the In-111 labelled protein is the clearing of circulating receptor-protein complex into the

system[59,284,301,305]. The TA_3/H mammary carcinoma is known to shed a high molecular weight epiglycanin, to which PNA binds[91]. The PNA-epiglycanin complex may then be trapped and cleared by the RES system. Antigen-antibody complex formation has been detected in human serum samples after the injection of In-111 19-9 antibody raised against the CA 19-9 antigen. The levels of circulating CA 19-9 in the patients dropped off rapidly after the administration of the antibody so that a minimum was reached in 30 min to 1 hr[284]. However, no obvious correlation can be made between hepatic uptake and the circulating antigen levels or the decrease in circulating antigen levels following antibody administration[284]. Other studies by Hagan *et al* [317] use athymic mice bearing tumor with different levels of carcinoembryonic antigen (CEA) secretion. The injection of In-111 and I-125 anti-CEA antibody resulted in a higher percent injected dose per gram of blood with the low CEA secreters than with the high CEA secreters. The mice with a high CEA tumor secreting rate had a markedly increased In-111 level in the liver while the liver accumulation of I-125 anti-CEA antibody was essentially the same regardless of the level of secreted CEA. Therefore, the formation of antigen-receptor complex may contribute to the uptake of the radiotracer in the liver. Probably both active antibody and antibody antigen complex are taken up by the liver[310].

Many researchers have attempted to reduce the non-target uptake of In-111 labelled proteins to enhance the tumor scintigraphy and to decrease the radiation dose to the patient[312-317]. The use of antibody fragments increased the excretion rate and reduced some non-target binding. Although lower blood and liver background was obtained, the concentration in the target tumor also suffered[312,313].

Goodwin *et al* [314] utilized a second antibody which is specific for the antibody injected. The blood background was decreased but a reciprocal increase in liver background was observed[314]. Local injection of antibodies subcutaneously or into body cavities limited the distribution of antibodies to other non-target organs but this approach is only suitable for a limited number of malignancies[315].

Recently, Goodwin *et al* [316] have developed two new methods using a reversibly labelled antibody-hapten complex and an In-111 labelled bifunctional hapten to reduce the blood background and non-tumor uptake of the radiolabelled antibodies. The first method involves the injection of the radiolabelled non-covalently binded antibody-hapten complexes into the animal followed by a nonradioactive hapten chase intravenously 1-3 hr prior to the optimum imaging time. The nonradioactive hapten will displace the radioactive hapten in the circulation which is then cleared rapidly through the kidneys[316].

In the second approach, the antibody was injected I.V. and allowed to reach the target tumor site by slow diffusion. The second step involves the injection of a matrix consisting of antigens covalently bound to a slowly diffusable serum protein (human transferrin) as a chase. The large size of the matrix prevents it from leaving the vascular space and diffusing into the target site. The antigen-antibody matrix is then removed rapidly through the RES system so that excess antibody was removed from the circulation, liver and kidney, but not from the tumor site. This is then followed by the I.V. injection of the In-111 labelled hapten which is rapidly diffusable to the tumor and the excess hapten is then cleared rapidly through the kidneys[316].

Biodistribution studies in a mouse model showed that a significant improvement in the tumor/non-target organ ratio can be obtained using this method. However, a relatively large amount of antibody (approximately 100 to 200 mg for a human) was needed to bind the tracer in the target. This is due to the relatively low binding constant of the antibody[316].

Newer chelates have been developed which can be effectively metabolized and cleared from the liver[274,317]. When complexed to an antibody, the blood and whole body background were lowered, but the target concentration were also affected due to the faster blood clearance[317]. This group of metabolizable chelates may be useful for the chelation with PNA since the liver metabolism of the lectins may play a major role in the accumulation of the In-111 DTPA-PNA in the liver.

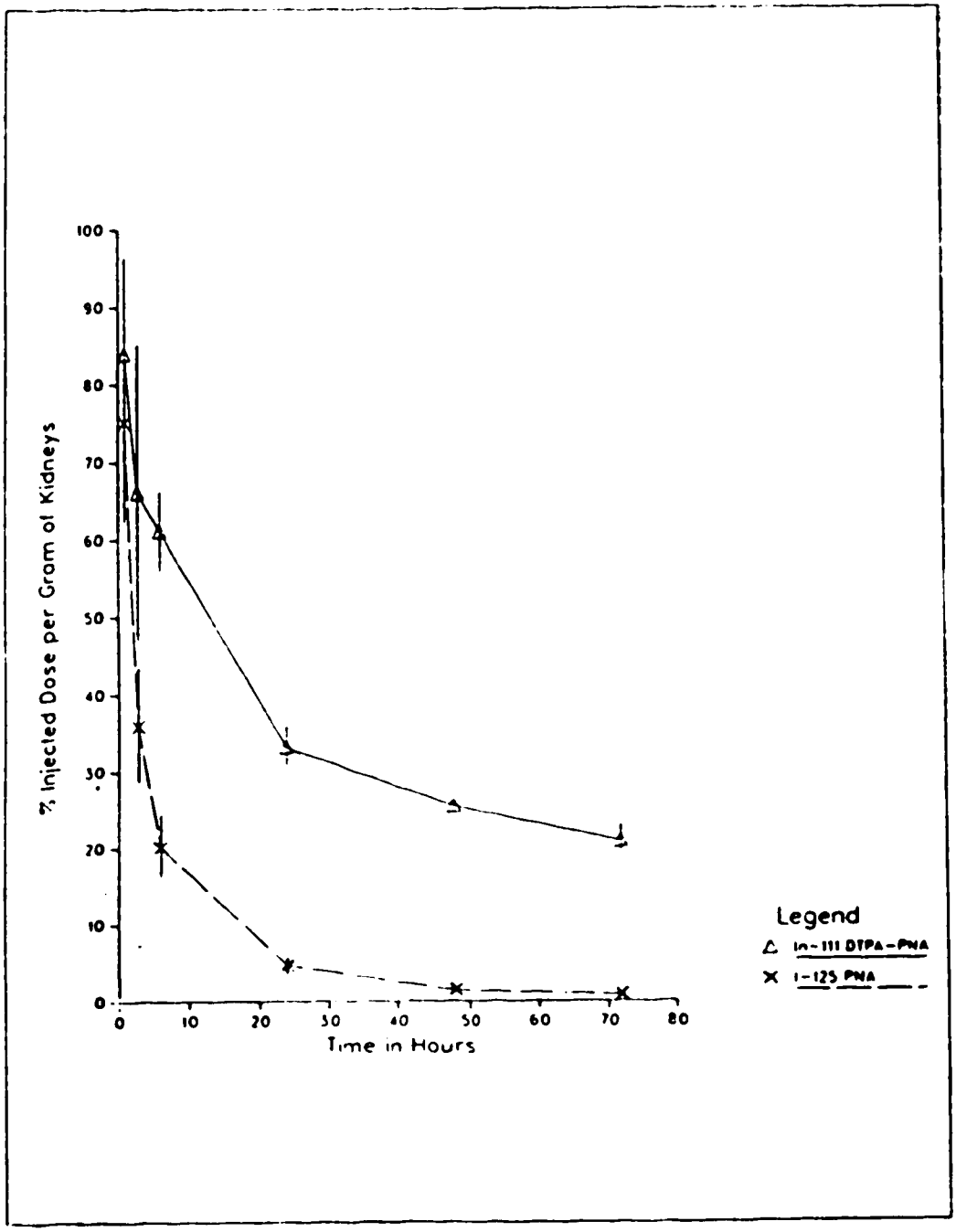
4.7.2.2 Kidneys

On a per gram basis, the kidneys accumulate the highest percentage of the injected dose of In-111 DTPA-PNA or I-125 PNA in the CAF_1/J mouse model (Table 28 and 29). The high kidney localization of radioiodinated PNA was seen previously using the TA_3/Ha tumor in strain AJ mice[104], and was also confirmed by the biodistribution studies in New Zealand White (NZW) rabbits and dogs[209]. A strain difference in the renal uptake and retention time of I-125 PNA was observed in mice. AJ and C57b mice behave similarly in their renal handling of I-125 PNA, while CBA/CAJ mice showed a lower accumulation of the radioiodinated lectin in the kidneys[209]. The CAF_1/J mice used in the present study demonstrated a similar percent injected dose per organ values in the kidney to the strain AJ and C57b mice in the early post injection time period, but the activity was cleared faster resulting in a lower percent injected dose per gram value at later time periods.

The strain difference in the renal elimination of the I-125 PNA may explain the discrepancies between the tumor uptake in the present study and the values reported by Eu *et al* [104] using a TA_3/Ha tumor model in strain AJ mice. The slightly lower uptake observed in this study may be a result of the faster renal clearance of the radiotracer thus decreasing the chance for localization within the tumor tissue.

When the renal uptake of I-125 PNA and In-111 DTPA-PNA is compared, a significant retention of the In-111 label in the kidneys is observed compared to the I-125 PNA(figure 20). The difference between the elimination kinetics of the two radiotracers may be due partly to the unique way PNA is handled in the kidney. PNA with a molecular weight of about 110,000 daltons is unlikely to be filtered by the glomerulus and studies using fluorescein labelled PNA showed no staining in the glomerulus thus confirming this assumption[209]. However, the proximal tubules of the cortex and a small percent of medullary cells are stained by FITC-PNA. Examination of the urine samples after the injection of radioiodinated PNA revealed that in the early time period, most of

Figure 20. The Renal Uptake of Radiolabelled PNA



the radioactivity excreted was protein bound and showed an affinity for asialo GM_1 synsorb indicating that biologically active PNA was excreted from the kidneys. At later time periods, the percent of protein bound radioactivity decreased probably due to the higher level of free radioiodide excretion[209].

Biogel P-100 analysis of the urine samples at later time periods post injection reveals the presence of intact PNA at the void volume, a lower molecular weight protein fragment and free radioiodide. This suggested that degradation of the PNA may have occurred during renal transit resulting in the formation of proteolytic fragments and/or free radioiodide[209].

With In-111 labelled proteins, an elevated kidney level is usually observed even when the corresponding radioiodinated protein does not show much kidney accumulation[293,294,297]. The urinary excretion of In-111 labelled antibody usually followed a biphasic elimination[296,305]. A rapid excretion of In-111 is observed in the first few hours post injection and is likely to be due to the clearing of In-111 DTPA which is a common radiochemical contaminant in the radiolabelled antibody preparation. The second phase of the elimination curve has a longer half-life and is probably responsible for the excretion of the metabolized antibody[296]. Radiochromatograms obtained by G-25 Sephadex ® chromatography of urine samples at various time periods revealed the change in composition of the radiolabelled material being excreted[214]. Excretion of In-111 DTPA is apparent at early time periods. However, at later time periods, there is a progressive increase in the abundance of a higher molecular weight species. When subjected to HPLC analysis, this species showed a lower molecular weight than the labelled antibody[214].

The difference between the renal excretion of In-111 DTPA-PNA and I-125 PNA is probably due to the different way the kidneys handled the two labels after metabolism of the intact PNA. Extensive *in vivo* metabolism of the PNA was evident when the composition of the radioactive species in the urine samples was examined[209]. In a rabbit animal model, as early as 1 hour post injection, the percent TCA precipitable

radioactivity in the urine starts to decrease as does the percent binding to the asialo GM_1 synsorb. This was probably due to the metabolism of the PNA molecule to smaller fragments and/or free radioiodide. This would explain the decrease in the asialo GM_1 synsorb binding as the PNA fragments did not maintain their T-antigen binding characteristics. After metabolism in the kidneys, the I-125 label was excreted in the urine[209]. However, in this present study, when the In-111 labelled PNA is metabolized in the kidneys, the In-111 released may be transchelated to metal binding proteins in the kidnev thus preventing it from being excreted from the kidneys. Metallothionein is a major metal binding protein found in the kidneys and may be responsible for the retention of In-111[318]. Thus the excretion of In-111 DTPA-PNA from the kidneys may be made up of two separate mechanisms, the elimination of intact PNA through a receptor mediated process and a slower clearance of metabolized products due to transchelation in the kidneys.

4.8 The Biodistribution of In-111 Citrate and In-111 DTPA

The data obtained from the biodistribution studies revealed that there is a significant amount of uptake into the TA_3/Ha tumor following the intravenous injection of In-111 DTPA-PNA. *In vivo* metabolism of the PNA molecule may result in the cleavage of the amide bond between the chelate and the PNA, thus releasing the free chelate (In-111 DTPA). In addition, transchelation of the In-111 label to other metal binding proteins (mainly transferrin) in the serum, may result in the formation of other In-111 protein complexes[284]. In order to determine if the localization of the radioactivity in the tumor site is due to either of these degradation products, the biodistribution of In-111 DTPA and In-111 citrate in the same animal tumor model was investigated.

4.8.1 The Biodistribution of In-111 DTPA

It was well known that DTPA-metal complexes are cleared rapidly through the kidneys and this allows them to be used as renal imaging agents[219]. This property of the In-111 DTPA complex is desirable as any radiochemical impurties in the preparation of In-111 DTPA-PNA if present as In-111 DTPA will be cleared from the kidneys shortly after the injection. This will eliminate the backgound radioactivity arising from this radiochemial impurty. The localization of In-111 DTPA in strain CAF_1/J mice bearing the TA_3/Ha tumor model model is presented in table 31. As seen in table 31, the whole body clearance of the radiotracer is rapid, with only 11.62% of the injected dose remaining at 1 hour post injection. After 24 hours post injection, less than 1% of the injected dose is retained in the body (figure 21). The kidneys are the major organ of excretion and also the only organ that showed any significant amount of uptake of the radiotracer (figure 22). Plasma clearance is also very rapid with only 0.20% of the injected dose remaining at 1 hour post injection. The rapid elimination of the In-111 DTPA from the body does not favour any significant uptake in the tumor site. Only 0.32% of the injected dose is accumulated per gram of tumor at 24 hours post injection compared to the 4.30% value obtained by the In-111 DTPA-PNA. This suggested that In-111 DTPA, if present in the In-111 DTPA-PNA preparation, does not contribute significantly to the uptake of In-111 radioactivity in the tumor site.

4.8.2 The Biodistribution of In-111 Citrate

The use of In-111 chloride or citrate for tumor localization has been well documented for some time[217,288,319]. It was shown that In-111 citrate was similiar to In-111 chloride in its *in vivo* properties and tumor uptake. This is thought to be due to the *in vivo* formation of In-111 transferrin from either of the two radiotracer which is then responsible for the uptake in tumor[288]. Farrar *et al* [288] studied the utility of In-111 transferrin as a tumor scanning agent in humans. After the i.v. injection of In-111 chloride, the plasma disappearance of the In-111 was shown to consist of two exponential components, with an initial rapid excretion phase

Table 31. Biodistribution of In-111 DTPA in Mice Bearing TA$_1$/Ha Tumor†

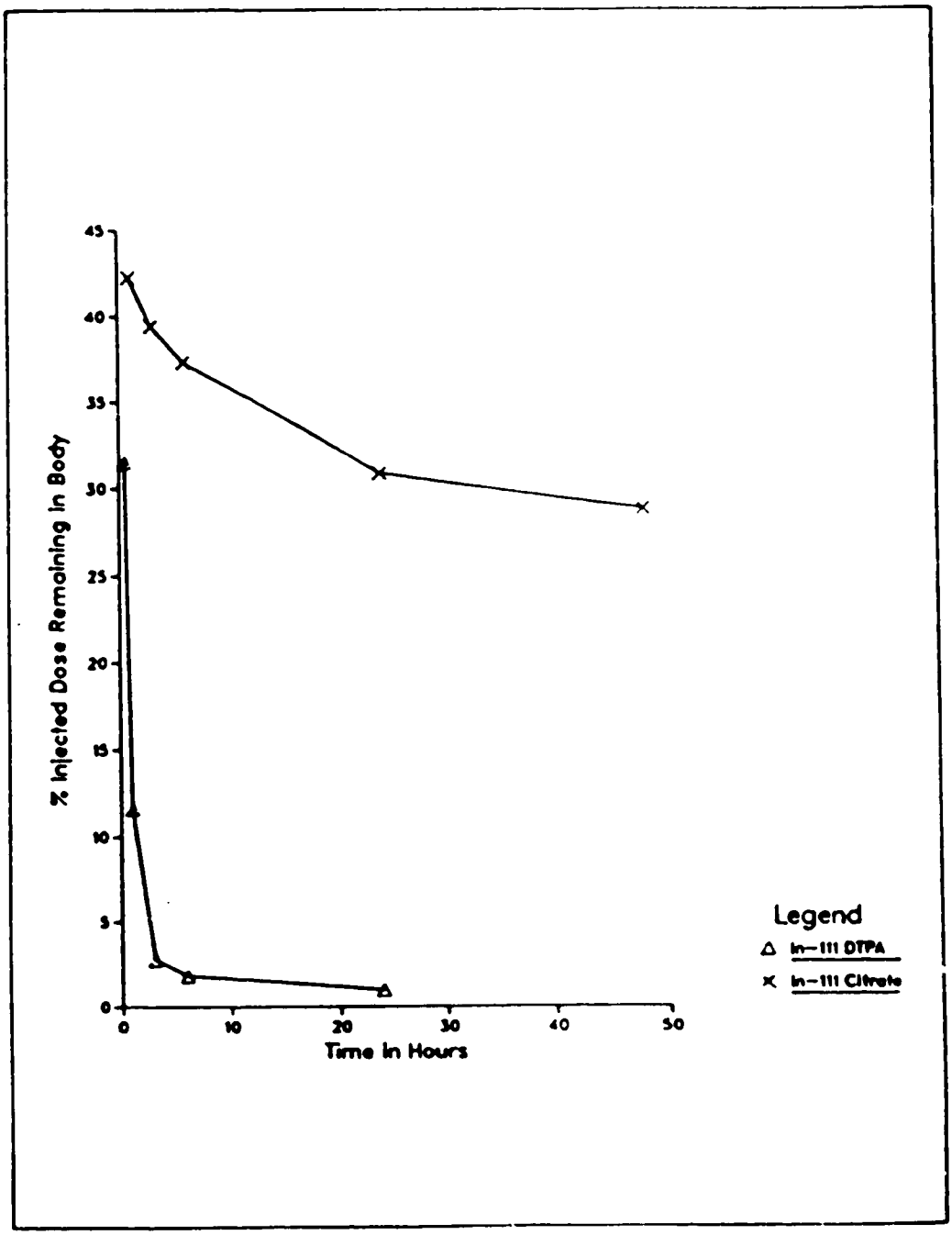
Organ		30 minutes n=3	1 hours n=4	3 hours n=4	6 hours n=3	24 hours n=4
Blood	% dose/g	1.47 ± 0.24	0.15 ± 0.02	0.02 ± 0.003	0.016 ± 0.002	0.011 ± 0.002
	% dose/organ	2.00 ± 0.34	0.20 ± 0.03	0.03 ± 0.01	0.02 ± 0.003	0.015 ± 0.004
Liver	% dose/g	0.48 ± 0.04	0.19 ± 0.02	0.15 ± 0.03	0.16 ± 0.02	0.08 ± 0.01
	% dose/organ	0.61 ± 0.07	0.25 ± 0.04	0.17 ± 0.01	0.18 ± 0.02	0.11 ± 0.02
	organ:blood	0.33 ± 0.05	1.38 ± 0.24	6.30 ± 1.45	10.23 ± 2.58	7.22 ± 1.64
Spleen	% dose/g	0.28 ± 0.04	0.08 ± 0.01	0.08 ± 0.02	0.07 ± 0.02	0.06 ± 0.01
	% dose/organ	0.10 ± 0.003	0.04 ± 0.01	0.02 ± 0.004	0.03 ± 0.01	0.02 ± 0.004
	organ:blood	0.19 ± 0.04	0.58 ± 0.14	3.48 ± 0.45	4.18 ± 0.59	5.31 ± 1.07
Kidneys	% dose/g	3.82 ± 1.01	1.52 ± 0.08	1.28 ± 0.20	1.31 ± 0.17	0.68 ± 0.03
	% dose/organ	1.32 ± 0.35	0.57 ± 0.06	0.45 ± 0.04	0.44 ± 0.03	0.24 ± 0.01
	organ:blood	2.61 ± 0.20	10.88 ± 2.34	55.41 ± 5.79	83.48 ± 12.46	59.30 ± 4.76
Tumor	% dose/g	1.71 ± 0.25	0.63 ± 0.03	0.41 ± 0.05	0.26 ± 0.03	0.32 ± 0.04
	organ:blood	1.18 ± 0.27	4.58 ± 1.38	17.51 ± 2.87	16.90 ± 3.45	27.91 ± 3.08
Lungs	% dose/g	0.96 ± 0.05	0.21 ± 0.02	0.11 ± 0.02	0.09 ± 0.01	0.09 ± 0.02
	% dose/organ	0.17 ± 0.02	0.03 ± 0.01	0.02 ± 0.003	0.01 ± 0.002	0.01 ± 0.003
	organ:blood	0.67 ± 0.08	1.45 ± 0.23	4.64 ± 1.08	6.12 ± 1.34	8.165 ± 2.08
Salivary	% dose/g	0.77 ± 0.09	0.25 ± 0.01	0.11 ± 0.02	0.10 ± 0.02	0.09 ± 0.01
	% dose/organ	0.12 ± 0.02	0.04 ± 0.01	0.02 ± 0.004	0.02 ± 0.002	0.01 ± 0.004
	organ:blood	0.54 ± 0.13	2.06 ± 0.45	4.61 ± 1.12	6.27 ± 2.07	8.03 ± 2.43

Continued...

Organ		30 minutes n=3	1 hours n=4	3 hours n=4	6 hours n=3	24 hours n=4
Stomach	% dose/organ	0.11 ± 0.04	0.03 ± 0.01	0.05 ± 0.01	0.02 ± 0.01	0.02 ± 0.004
	organ:blood	0.23 ± 0.12	0.89 ± 0.21	6.45 ± 1.04	4.64 ± 0.58	3.78 ± 0.72
% injected dose remaining in carcass		27.10 ± 3.68	10.58 ± 1.63	2.02 ± 0.31	1.12 ± 0.08	0.57 ± 0.06
% dose remaining in body except tumor		31.53 ± 3.59	11.62 ± 1.67	2.77 ± 0.37	1.83 ± 0.09	0.98 ± 0.08

† Mean ± S.D. % of injected dose per gram of tissue and entire organ following i.v. injected of 74KBq In-111 DTPA.

Figure 21. The Total Body Clearance of In-111 DTPA and In-111 Citrate in CAF_1/J Mice



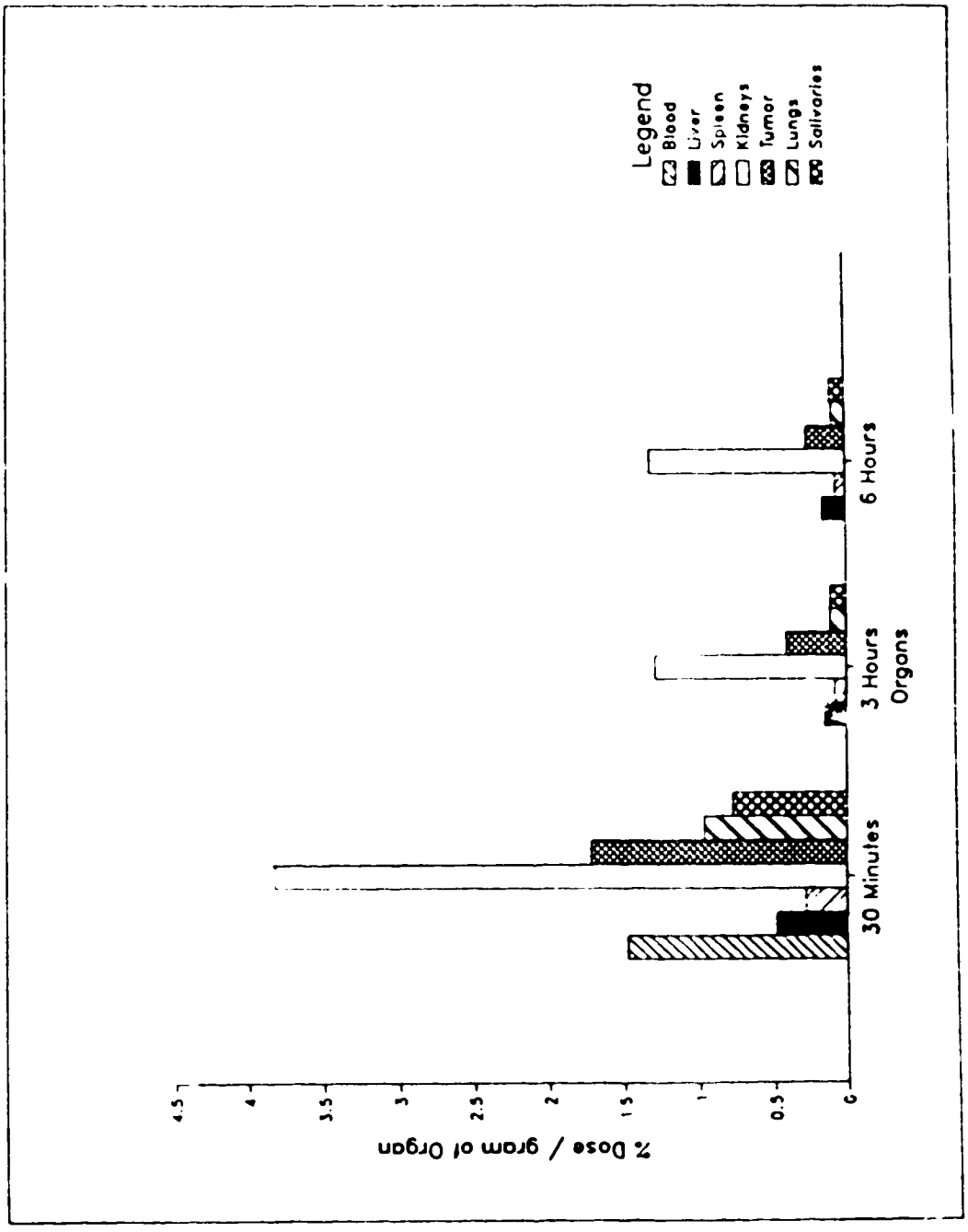


Figure 22. The Biodistribution of In-111 DTPA in TA_3/Ha Tumor Mouse Model

with a T-1/2 of about 4 hours, followed by a slow elimination phase with a T-1/2 of about 30 hours. The tumor:thigh activity increased with time reaching a plateau between 48 and 72 hours. There was also a high level of uptake in the bone marrow which made it difficult to visualize tumors in the thorax and mediastinum[288].

The mechanism of the localization of In-111 citrate in the tumor is still not very well understood at the present. It is generally believed that diffusion of the radiotracer into the tumor followed by intracellular entrapment of the radionuclide is responsible for its uptake in the tumor[...].

The biodistribution of In-111 citrate in the TA_3/Ha mouse tumor model is presented in table 32. The biodistribution of In-111 citrate is very different from that of In-111 DTPA-PNA (table 29). The whole body clearance of In-111 citrate appears to be biphasic, with an rapid clearance within the first hour, followed by slower elimination of the radiotracer, with 30.87% and 28.79% of the injected dose remaining in the body at 24 and 48 hours post injection. The major organs responsible for the uptake of In-111 citrate are the liver, spleen and kidneys. A considerable amount of radioactivity still remains in the circulation at 24 and 48 hours post injection. Uptake in the tumor is not very high, with only 1.55% of the injected dose per gram of tumor compared to the 4.40% per gram obtainable with In-111 DTPA-PNA. This low level of tumor accumulation coupled with the high level of background radioactivity gives rise to a low tumor to blood and tumor to non-target organ ratio. These findings are in agreement of previous works with In-111 citrate which showed a high background uptake of this radiotracer[287,288,319].

The relatively high tumor uptake and rapid blood clearance observed with In-111 DTPA-PNA suggested that this radiotracer may localize in the tumor *via* a receptor mediated process and is not due to the non-specific trapping of In-111 transferrin in the tumor as a result of serum transchelation.

Table 32. Biodistribution of In-111 Citrate in Mice Bearing TA$_3$/Ha Tumor†

Organ		1 hour n=3	3 hours n=4	6 hours n=3	24 hours n=4	48 hours n=4
Blood	% dose/g	5.59 ± 0.88	4.24 ± 0.62	2.8[illegible] ± 0.30	1.97 ± 0.09	0.73 ± 0.07
	% dose/organ	7.68 ± 1.98	5.92 ± 1.08	3.79 ± 0.62	2.65 ± 0.11	1.03 ± 0.09
Liver	% dose/g	1.75 ± 0.12	1.80 ± 0.12	1.96 ± 0.07	2.74 ± 0.10	2.84 ± 0.19
	% dose/organ	2.60 ± 0.45	2.75 ± 0.35	2.84 ± 0.44	3.96 ± 0.23	4.68 ± 0.58
	organ:blood	0.31 ± 0.05	0.45 ± [illegible]02	[illegible]1 ± 0.07	1.29 ± [illegible]10	3.92 ± 1.02
Spleen	% dose/g	1.78 ± 0.23	2.29 ± [illegible]	[illegible] ± 0.12	2.97 ± 0.39	1.97 ± 0.05
	% dose/organ	1.73 ± 0.09	1.26 ± 0.16	[illegible]5 ± 0.04	1.44 ± 0.24	1.66 ± 0.04
	organ:blood	0.32 ± 0.05	0.55 ± 0.03	[illegible] ± 0.06	1.37 ± 0.14	2.71 ± 0.19
Kidneys	% dose/g	10.07 ± 3.65	11.96 ± 3.72	19.30 ± 2.87	10.44 ± 1.78	8.59 ± 1.96
	% dose/organ	3.52 ± 0.68	4.47 ± 1.29	6.72 ± 2.21	3.67 ± 0.68	3.45 ± 0.59
	organ:blood	1.87 ± 0.35	3.19 ± 1.01	7.41 ± 1.24	5.15 ± 0.89	11.85 ± 3.67
Tumor	% dose/g	1.90 ± 0.18	1.23 ± 0.17	2.09 ± 0.[illegible]	1.55 ± 0.22	1.39 ± 0.09
	organ:blood	0.31 ± 0.02	0.32 ± 0.04	0.7[illegible]	0.75 ± 0.12	1.91 ± 0.10
Lungs	% dose/g	3.43 ± 0.45	3.10 ± 0.26	2.[illegible]	2.53 ± 0.16	1.98 ± 0.09
	% dose/organ	0.56 ± 0.07	0.59 ± 0.02	0.[illegible]	0.73 ± 0.03	0.40 ± 0.01
	organ:blood	0.61 ± 0.07	0.80 ± 0.03	1.07 ± [illegible]	2.31 ± 0.01	2.72 ± 0.23
Salivary	% dose/g	1.74 ± 0.25	1.99 ± 0.30	1.92 ± 0.05	1.71 ± 0.04	1.65 ± 0.13
	% dose/organ	0.25 ± 0.01	0.26 ± 0.02	0.26 ± 0.02	0.24 ± 0.02	0.23 ± 0.01
	organ:blood	0.32 ± 0.03	0.50 ± 0.07	0.68 ± 0.03	0.83 ± 0.02	2.28 ± 0.65

Continued...

Organ		1 hour n=3	3 hours n=4	6 hours n=3	24 hours n=4	48 hours n=4
Stomach	% dose/organ	0.15 ± 0.01	0.19 ± 0.01	0.16 ± 0.02	0.12 ± 0.01	0.12 ± 0.01
	organ:blood	0.08 ± 0.01	0.14 ± 0.03	0.19 ± 0.03	0.17 ± 0.01	0.68 ± 0.02
% injected dose remaining in carcass		26.75% ± 2.78	23.90 ± 3.64	22.10 ± 2.56	18.06 ± 1.22	17.22 ± 0.56
% dose remaining in body except tumor		42.24 ± 5.52	39.34 ± 5.69	37.35 ± 3.65	30.87 ± 2.34	28.79 ± 1.29

† Mean ± S.D. % of injected dose per gram of tissue and entire organ following i.v. injected of 74KBq In-111 Citrate

4.9 Whole Body Scintigraphy

It has been demonstrated that I-125 PNA injected intravenously can clearly delineate the TA_3/Ha adenocarcinoma and selected tumor variants in the AJ mouse model[104]. Whole body gamma scintigraphic imaging using In-111 DTPA-PNA and In-111 citrate were compared to the images obtained from the I-125 PNA to investigate the potential of these agents for the radioimaging agent in the detection of T antigen expressing tumors. Strain CAF_1/J mice bearing a s.c. nplanted solid tumor in the right flank were used as the animal tumor model. The results obtained from the biodistribution studies demonstrated that the In-111 DTPA-PNA has a higher percent dose per gram than I-125 PNA, however, the background activity associated with the In-111 tracer is also significantly higher than that seen with I-125 PNA.

The whole body gamma scintigraphic images obtained from the tumor bearing mice are presented in figures 23, 24 a 25. *In vivo* deiodination is a major limitation for the use of radioiodinated proteins in radioscintigraphic studies. The presence of free radioiodides after the I.V injection of I-125 PNA is apparent as early as 6 hours post injection and appeared as a "h spot in the thyroid. In these studies, no pre-treatment with Lugol's solution was used. Also, high kidney activity and some stomach activity is visible. Tumor uptake is also visible with low background acti in the liver and lungs. The background radioactivity continues to clear from the body so that at 72 hours post injection, only the thyroid, the kidneys and the tumor are visible on the scan. The clear delineation of the TA_3/Ha tumor without background substration techniques confirms the utility of the radioiodinated lectin in the scintigraphic detection of T-antigen expressing tumor in the animal model.

Figure 24 represents the whole body image taken when the tumored mice are injected with In-111 DTPA-PNA. The high background activity in the early time period was expected from the biodistribution data which showed high uptake in the liver and the kidney. The high background radioactivity decreases the contrast between the tumor and the non-target tissue but the tumor site can still be clearly identified. No thyroid radioactivity is observed with

Figure 23. Static Gamma Scintigraphy of Intravenously Injected I-125 PNA in a Mouse Bearing a Solid TA_3/Ha Tumor in the Right Flank

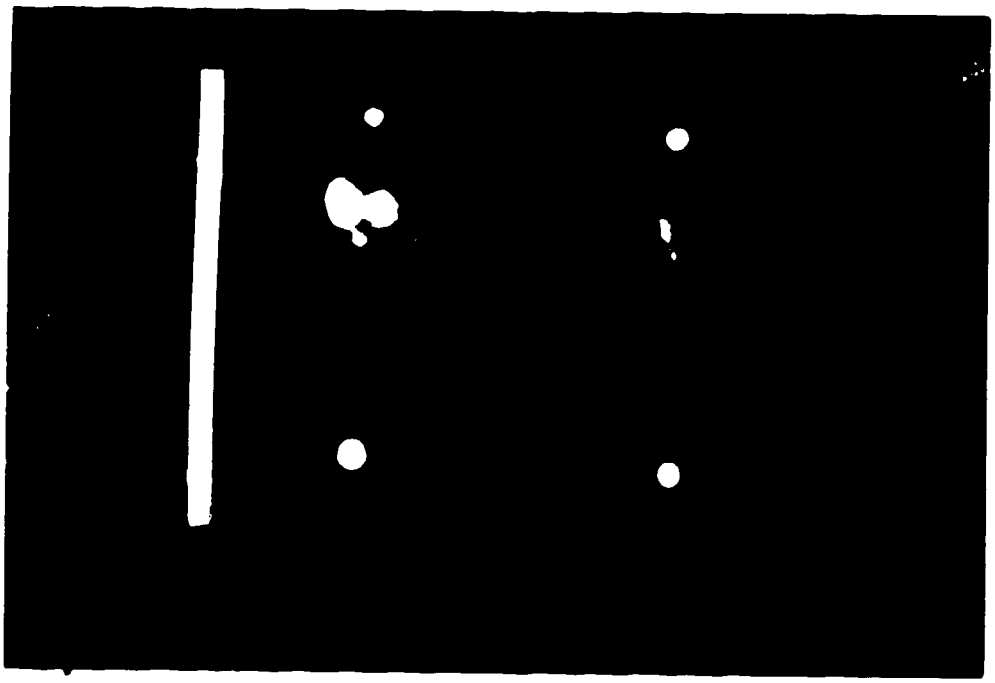


Figure 24. Static Gamma Scintigraphy of Intravenously Injected In-111 DTPA-PNA in a Mouse Bearing a Solid TA_3/Ha Tumor in the Right Flank

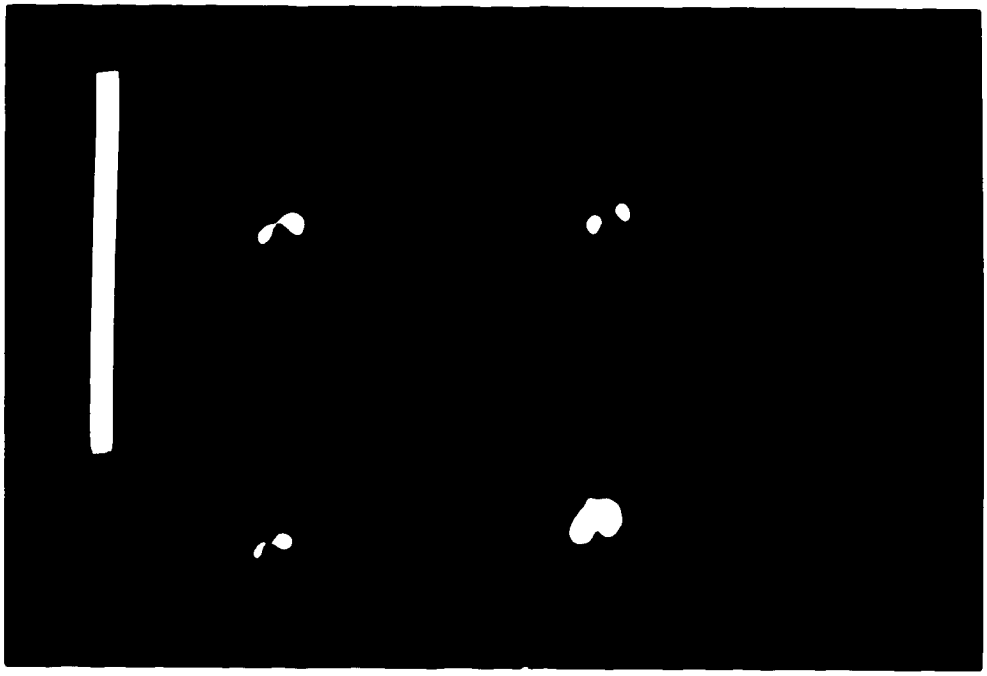
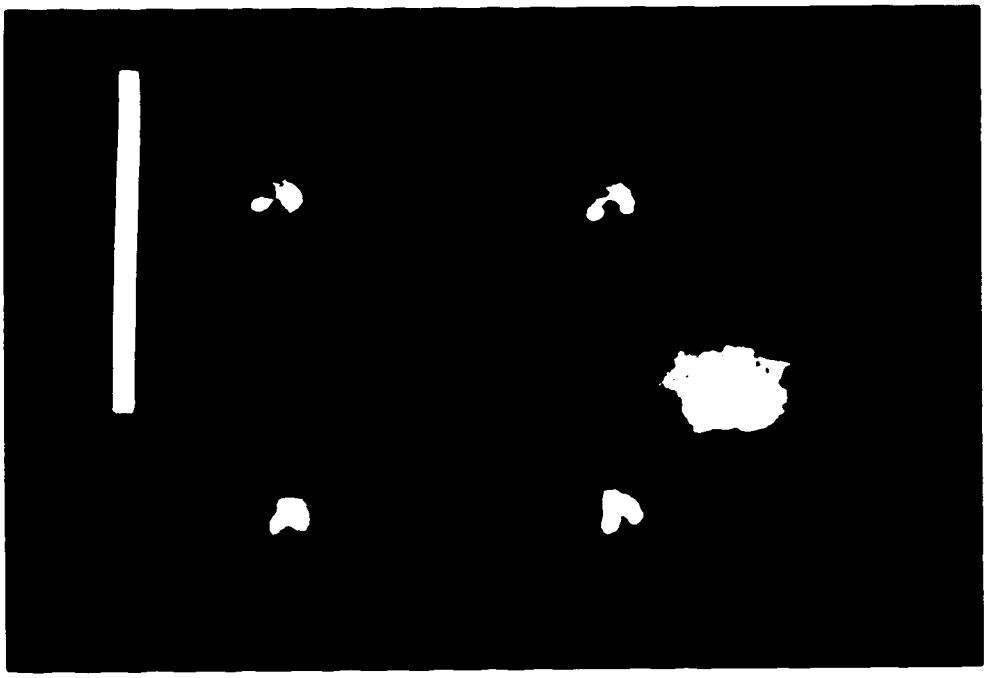


Figure 25. Static Gamma Scintigraphy of Intravenously Injected In-111 Citrate in a Mouse Bearing a Solid TA_3/Ha Tumor in the Right Flank



In-111 DTPA-PNA as opposed to I-125 PNA showing the different handling of the two isotopes by the mice. The background radioactivity is cleared slowly from the body, however, the liver and kidneys continue to show a high concentration of the radiotracer. At 72 hours post injection, only the liver, kidneys and the tumor are clearly delineated in the scan. However, due to the decrease in the tumor to liver and tumor to kidney ratio, the quality of the scan for the tumor did not improve with time. The high concentration of the radiotracers in the liver may also make the detection of specific liver variant metastases difficult.

On the whole, the two differently radiolabelled PNA preparations both showed clear delineation of the TA_3/Ha adenocarcinoma in the CAF_1/J mice without the need of background subtration techniques. However, they both have their advantages and disadvantages. The In-111 DTPA-PNA did not present with the same problem as *in vivo* deiodination, but the higher background radioactivity decreased the contrast between the target and non-target organs.

The whole body gamma scintigraphy of tumor bearing mice when injected with In-111 citrate is presented in figure 25. High background radioactivity is observed in the body with no apparent improvement up to 72 hours post injection of the radiotracer. This reflects the general non-specific interaction that is usually observed with group III B metal ions such as gallium and indium when injected intravenously[320]. High kidney radioactivity is seen in the early post injection time but the liver continues to accumulate the radioactivity and becomes more prominent in the later time periods. Some localization is evident in the tumor site but the high background radioactivity makes the exact delineation of the tumor site somewhat difficult.

The scans obtained from the In-111 citrate again reassured that the clear delineation of the T-antigen expressing tumor with radiolabelled PNA were indeed a receptor-specific process. The lack of diffuse whole body distribution of the In-111 DTPA-PNA also demonstrates the *in vivo* stability of the lectin and that the tumor uptake is mainly due to the lectin and T-antigen receptor interaction. More work has to be done to try to limit the uptake

in non specific organs in order to increase the utility of the In-111 labelled PNA. The recent development of chelates which are metabolized in the liver may help to minimize such uptake in the liver[274].

Recently, Zalutsky and Narala [321] have reported a method for the radiohalogenat.. of proteins which decreased thyroid uptake of radiodine. This would eliminate the ba.. radioactivity due to the accumulation of free radioiodides in non-target organs. T.. *vivo* dehalogenation of radioiodinated proteins is presumably due to the structural similarity between the radioiodophenyl groups and the thyroid hormones. The N-succini.. l ester of 3-tri-*n*-butylstanyl benzoate (ATE) was radioiodinated bearing the iodine in the *meta* positions on the phenyl ring. This arrangement of the halogen does not involve substitution of the halogen *ortho* to a hydroxyl group on an aromatic ring which is the mechanism for other commonly used radioiodination methods[321]. The radioiodinated ATE is then coupled to the pr..tein via an amide linkage. The biodistibution of I-125-goat IgG labelled using the ATE reagent showed a significant decrease in the thyroid uptake of radioiodine from day 1 to 8 post injection when it is compared to I-131 goat IgG labelled with the iodogen method. No significant difference between I-125 and I-131 uptake were observed at other times[321]. Such an approach may possibly be advantageous with radioiodinated PNA.

4.10 Dynamic Kidney Activity Time Curve in NZW Rabbits

The renal excretion of radioiodinated PNA is believed to be a receptor mediated process by the proximal tubular cells of mouse, rabbit and dog[209]. From the data obtained from the biodistribution studies, it was observed that In-111 DTPA-PNA was accumulated in the kidneys to a greater extent than I-125 PNA. The renal kinetics of the uptake of these two radiotracers were studied more carefully by examining the dynamic renal activity time curve using NZW rabbits as the test model.

The dynamic renal activity time relations for In-111 DTPA-PNA and I-131 PNA was revealed in figures 26 and 27. The time to peak and estimated percent injected dose at peak

Figure 26. Dynamic Renal Activity Time Curve of Intravenously Injected In-111 DTPA-PNA in NZW Rabbits

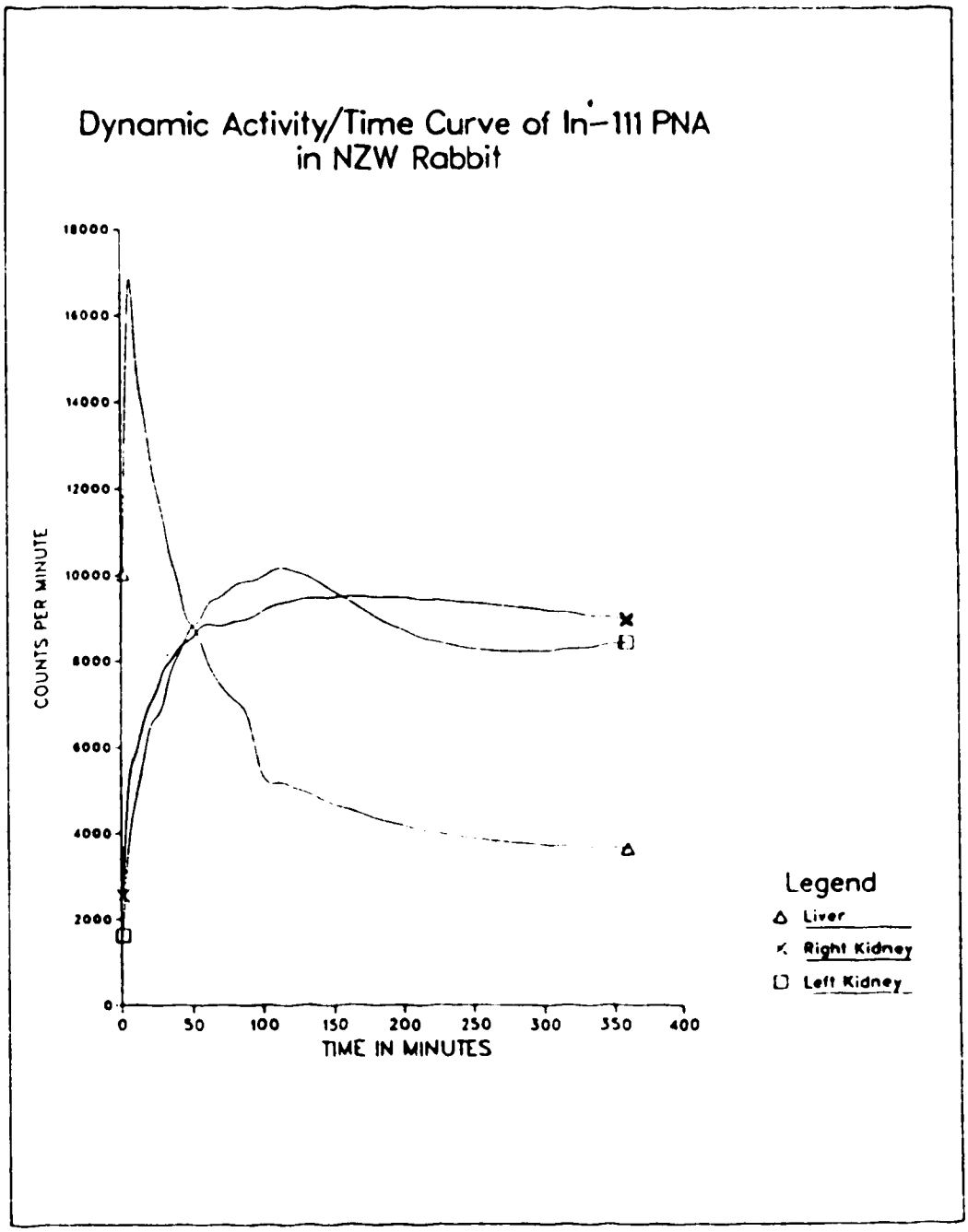
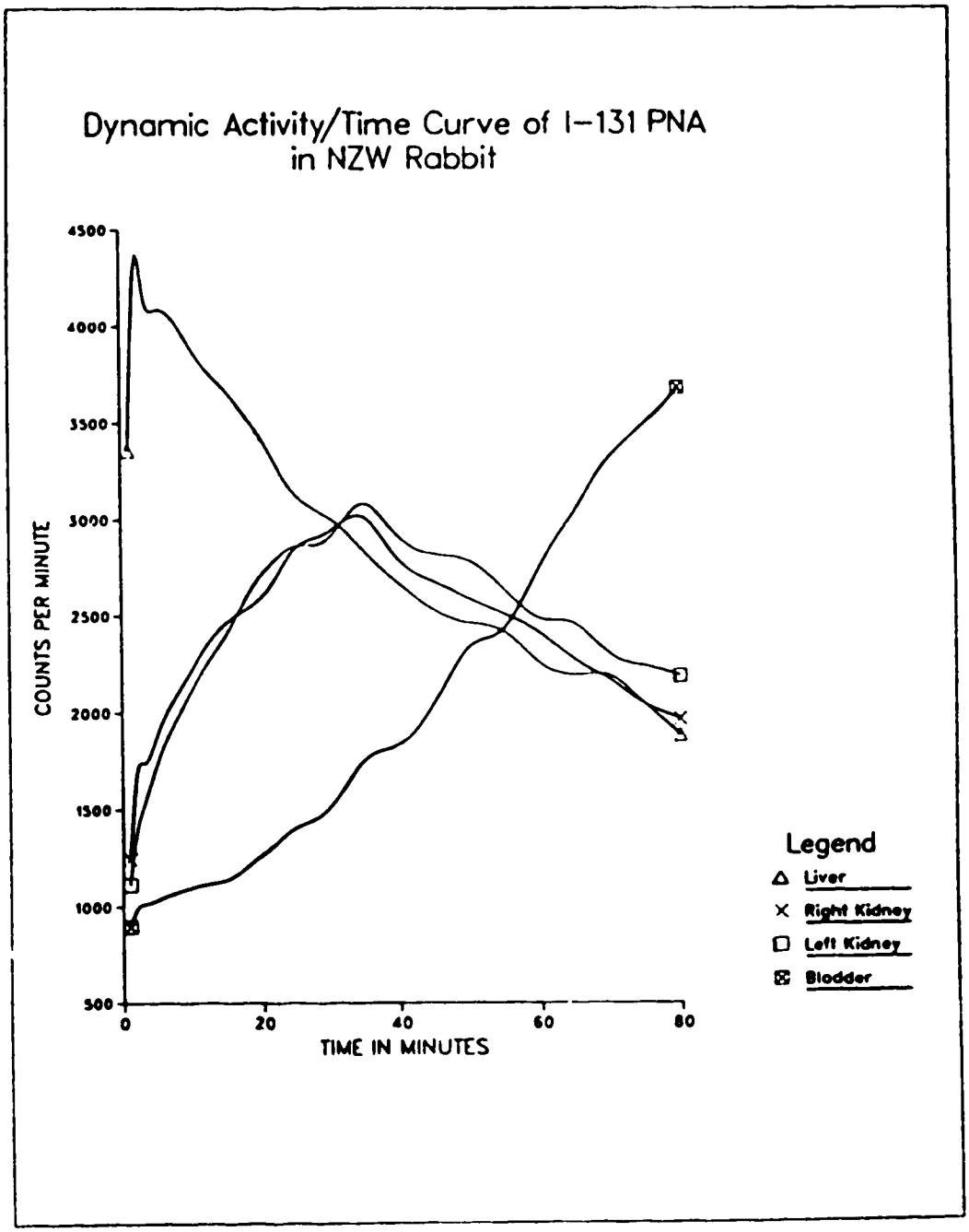


Figure 27. Dynamic Renal Activity Time Curve of Intravenously Injected I-131 PNA in NZW Rabbits



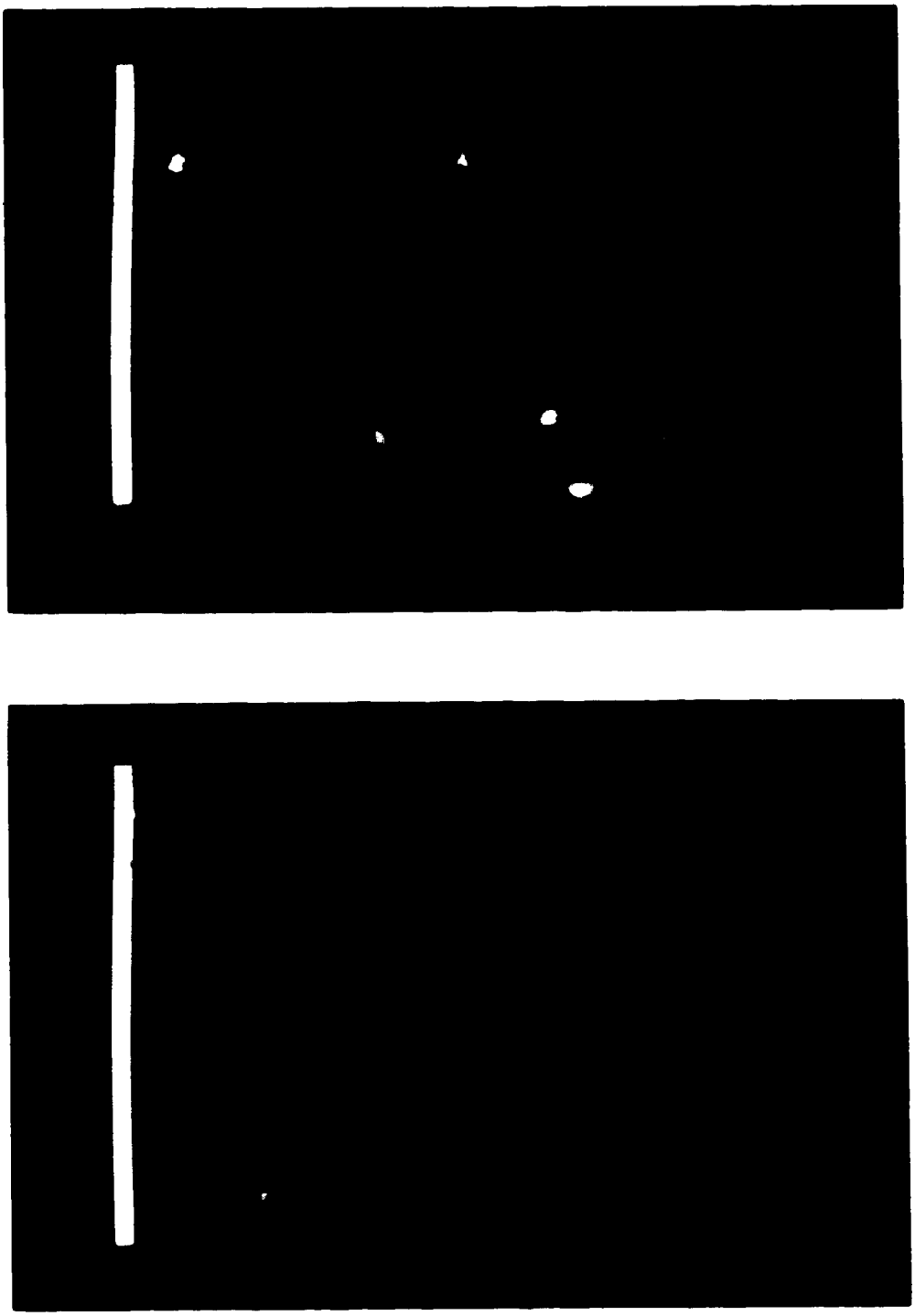
were approximately 35 minutes and 16% respectively for I-131 PNA. Static scintigrams up to 80 minutes post I.V. injection revealed the accumulation of activity in the kidney and bl: .der with rapid clearance from the liver (Figure 28). The renogram obtained for In-111 DTPA-PNA is quite different. The radioactivity in the kidneys continues to accumulate, reaching a plateau level in about a hour. From then on, the radioactivity is cleared slowly from the kidneys. The percent injected dose at peak was about 26%. The liver and kidneys are the two major organs revealed in the static scintigrams confirming the biodistribution and imaging studies in mice. The In-111 radioactivity is cleared from the liver more rapidly than from the kidneys, so that at 90 minutes post injection, the kidneys become the major organ visualized in the scintigrams.

The high kidney retention of In-111 DTPA-PNA is probably due to the accumulation of the metabolized fragments of the PNA in the kidneys. Iron binding proteins in the kidneys may effectively tie up any free indium which is produced along the metabolic process[311]. To determine if intact PNA was actually excreted from the kidneys, a finding which is consistent for radioiodinated PNA, urine analysis was performed by collecting the urine samples after the I.V. injection of In-111 DTPA-PNA in Sprague Dawley rats.

4.11 The Urinary Excretion of In-111 DTPA-PNA

The kinetics of urinary excretion of In-111 DTPA-PNA is quite different from that of the radioiodinated PNA as shown in the renograms. In order to further investigate the nature of the species that is being excreted, urinary samples from Sprague-Dawley rats, which were injected intravenously with In-111 DTPA-PNA, were collected and analyzed by gel filtration chromatrography and binding studies with synthetic T-antigen. The rats were kept in a metabolic cage after the injection of In-111 DTPA-PNA and the urine was collected as a batch pool. An aliquot of the urine sample was then passed through a Bio-Gel P-300 (exclusion limit 300,000 daltons) column and the eluted peaks were collected separately. The fraction corresponding to the molecular weight of monomeric PNA was tested for the *in vitro*

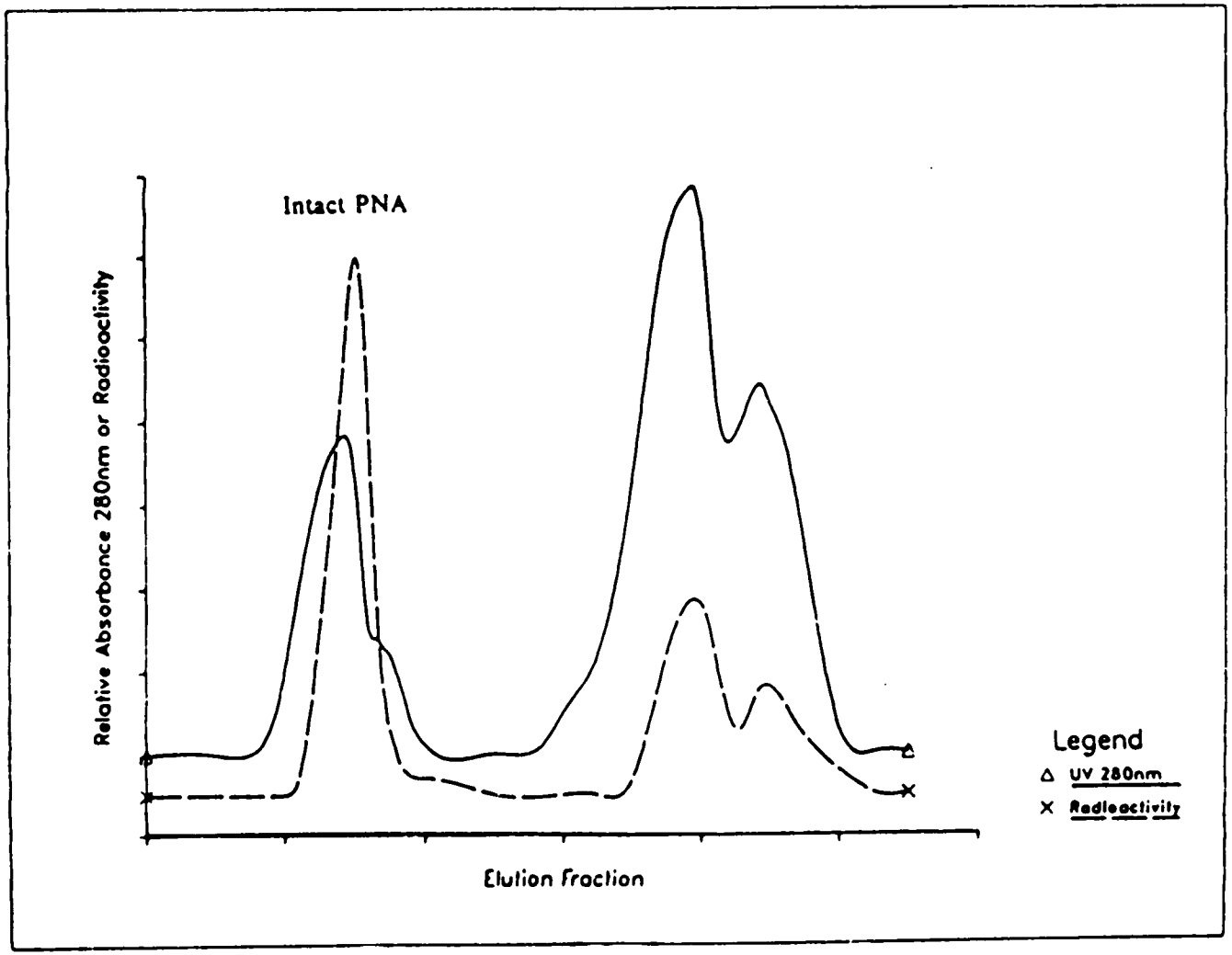
Figure 28. Static Gamma Scintigraphy of Intravenously Injected In-111 DTPA-PNA and I-131 PNA in NZW Rabbits



binding to T-Synsorb to estimate the T-antigen binding capacity of the excreted product.

The UV absorption and radioactivity profile of the urine sample following the elution through a Bio-Gel P-300 column is presented in figure 29. A large portion of the radioactivity was associated with the peak which elutes with a molecular weight similar to monomeric PNA. When this fraction was incubated with T-Synsorb, a binding value of 25% was observed which was only slightly less than the value observed with freshly labelled In-111 DTPA-PNA. These results suggested that biologically active PNA molecules are contained in this urine fraction. This observation confirms the reports that the biologically active I-125 or I-131 PNA were excreted through the kidneys of a few species of animals studied[209]. Two smaller peaks containing In-111 radioactivity were also eluted at the lower molecular weight fraction. The exact nature of these two species had not been determined but they did not possess any affinity for the T-antigenic structure as determined by binding to the T-Synsorb. The relatively large proportion of high molecular weight In-111 excretion products may suggested than the smaller metabolized fragments of PNA may be retained inside the kidneys probably due to the action of metal binding proteins in the kidneys. Examination and identification of the In-111 associated species in the extracts obtained from the kidney homogenate may yield more important information on the subject.

Figure 29. Analysis of the Renal Excretion Products in Rat Urine by Bio-Gel® P-200 Gel Filtration after the I.V. Injection of In-111 DTPA-PNA



5. SUMMARY AND CONCLUSIONS

1. The conditions and parameters for the radiolabelling of PNA with In-111 through a DTPA conjugation followed by In-111 chelation were investigated. The In-111 DTPA-PNA product was then characterized and compared with radioiodinated PNA in several *in vitro* and biological systems.
2. The conjugation of DTPA to PNA can be easily performed in a one step reaction by adding the PNA solution into a DTPA dianhydride coated reactivial. The DTPA-PNA can be purified by a single passage through a Bio-Gel® P-200 column. Aliquots of the purified DTPA-PNA solution after freeze-drying will result in a convenient kit-form preparation.
3. The efficiency of the DTPA-PNA conjugation reaction is affected by a variety of conditions, including the pH of the buffer used, the concentration of the PNA solution and the molar ratio between PNA and DTPA dianhydride. The optimal pH for the conjugation reaction is found to be 8.5 and a high PNA concentration (20 mg/ml) favors the conjugation yield. Increasing the molar ratio between DTPA dianhydride and PNA lowers the conjugation efficiency but the average number of DTPA residues attached per PNA molecule increases.
4. The radiolabelling of the DTPA-PNA with In-111 can be easily performed by mixing the required amount of In-111 citrate with the DTPA-PNA kit preparation. The labelling reaction is completed within 15 minutes. The unbound In-111 citrate can be separated from the In-111 DTPA-PNA by using a minicolumn (Bio-Gel® P-100) centrifugation procedure. About 80-85% of the In-111 DTPA-PNA is recovered from the column with over 95% radiochemical purity as determined by TLC.
5. The radiolabelling efficiency of DTPA-PNA is determined by the average number of chelates attached per PNA molecule and the amount of In-111 citrate used in the reaction. The radiolabelling efficiency increases with increasing level of chelate attachment. The radiolabelling efficiency also decreases slowly with the addition of large

amount of In-111 citrate.

6. A typical DTPA-PNA kit can be produced by reacting PNA (20 mg/ml) with DTPA dianhydride at a starting DTPA/PNA molar ratio of 10:1 in 0.2M phosphate buffer pH 8.5. The purified DTPA-PNA from this reaction contains an average of 3.2 DTPA/PNA molecule. In-111 radiochelation efficiency of about 90-95% is routinely achieved with specific activities between 160 - 200 KBq/μg of the labelled PNA.
7. The In-111 DTPA-PNA preparation is stable after 72 hours of storage at room temperature with over 98% of the original radioactivity being retained on the protein.
8. The In-111 DTPA-PNA is also quite resistant to challenging with an 10:1 molar excess of EDTA and DTPA or against transchelation in serum. Over 90% of the In-111 label was retained on the PNA after 48 hours of incubation with the chelating agents. A gradual loss of the In-111 label from the PNA is observed when it is incubated with serum. The In-111 DTPA-PNA still retains 88% of its T-Synsorb binding capacity after 48 hours of *in vitro* incubation with serum.
9. The In-111 DTPA-PNA preparation obtained retains its T-antigen binding ability. Binding to neuraminidase-treated red blood cells, T-antigen expressing tumor cell lines and the synthetic T-antigen (T-Synsorb) are all comparable to that obtained with I-125 PNA. The binding can all be readily inhibited by 0.05M galactose confirming the specificity of the binding.
10. Biodistribution studies with In-111 DTPA-PNA in mice bearing the TA_3/Ha tumor at various time periods revealed high tumor uptake of the radiotracer but uptake in various non-target organs, especially the liver and kidneys, are also very high. Compared to I-125 PNA, In-111 DTPA-PNA has a slower whole body clearance and a higher uptake in the liver and kidneys. Thus, although the percent injected dose/gram of tumor is higher with In-111 DTPA-PNA than with I-125 PNA, the tumor to blood and tumor to non-target organ ratio did not show much improvment with time.
11. The biodistribution of In-111 DTPA and In-111 citrate in the TA_3/Ha tumor model

confirmed the specificity of the uptake of In-111 DTPA-PNA in the same animal tumor model. While, In-111 DTPA is excreted rapidly through the kidneys, the In-111 citrate showed a diffuse pattern of localization that does not resemble the uptake of In-111 DTPA-PNA.

12. The rate of clearance of the radiolabels in the liver and kidneys are markedly different for I-125 PNA and In-111 DTPA-PNA. The In-111 label is retained longer in these organs probably due to the interaction with other metal binding proteins present in these organs.
13. The biodistribution of I-125 PNA and I-131 DTPA-PNA [illegible] imilar suggesting that the high liver uptake of In-111 DTPA-PNA may be attribu[illegible]he In-111 label itself
14. Whole body scintigraphic imaging with In-111 DTPA-PNA in CAF_1/J mice bearing solid TA_3/Ha tumor revealed good delineration of the tumor at 6 hours post injection without the need for blood background subtraction. However, the images fail to improve significantly with time due to the high non-target uptake of the radiotracer. No uptake in the thyroid is observed as opposed to that of I-125 PNA.
15. The kinetics of the accumulation of the radiolabelled PNA in the kidneys of NZW rabbits are different for In-111 DTPA-PNA than for I-131 PNA. I-131 PNA reached the peak kidney level in about 35 minutes post injection and than began to decline. The In-111 DTPA-PNA reached a plateau in about an hour and thereafter the radioactivity level remained elevated for a long period of time.
16. Intact In-111 DTPA-PNA was observed in the urine of Sprague Dawley rats over the first 24 hours. This supports the receptor mediated excretion mechanism proposal by Boniface *et al* [209]. Other smaller molecular weight forms which do not possess affinity for the T-antigen are also observed in the urine sample. These forms may result from the *in vivo* metabolic breakdown of In-111 DTPA-PNA.

BIBLIOGRAPHY

1. Paterson, A.H.G. and McReady, V.R. "Tumor Imaging Radiopharmaceuticals." *Br. J. Radiol* **48**: 520, 1975.

2. Winchell, H.S. "Mechanisms for Localization of Radiopharmaceuticals in neoplasm." *Sem, Nucl. Med.* **6**: 371, 1976.

3. O'Mara, R.E. and Mozley, J.M. "Current Status of Brain Imaging." *Sem. Nucl. Med.* **1**: 7, 1971.

4. Marty, R., Denney, J.D., McKamey, M.R., *et al.*, "Comparsion of Sr-85, Sr-87m, F-18 and Tc-99m Labelled Phosphates for Bone Scanning." *CRC Crit. Rev. Clin. Radiol. Nucl. Med.* **6**: 403, 1978.

5. Rothschild, M.A., Oratz, M.O. and Schreiber, S.S. "Comments on Radionuclide Hepatic Scanning." *Seminars In Liver Diseases* **2**: 29, 1982.

6. Lopez-Majano, V. and Alvarez-Cervera, J. "Neoplasm Localization with Radionuclides." *Eur. J. Nucl. Med.* **4**: 313 ,1979.

7. Haynie, T.P. and Glenn, H.J. "Current Status of Tumor Imaging." in *Tumor imaging : The radiochemical detection of cancer.* Burchiel, S.W. and Rhodes, B.A. (eds), Masson publishing Inc., USA, 1982. p.17.

8. Paterson, A.H.G. and McReady, V.R. "Clinical and Experimental Studies of Selenium-75 Labelled Compounds." in *Tumor localization with radioactive agents.* International atomic energy agency, Vienna, 1976. p.63.

9. Taylor, D.M. and McReady, V.R. "Labelled Antibiotics as Tumor Localizing Agents." in *Tumor localization with radioactive agents.* International atomic energy agency, Vienna, 1976. p.103.

10. Bekerman, C., Hoffer, P.B. and Bitran, J.D. "The Role of Gallium-67 in the Clinical Evaluation of Cancer." *Sem. Nucl. Med.* **15**: 72, 1985.

11. Edwards, C.L. and Hayes, R.L., "Tumor Sacnning with Gallium-67 Citrate." *J. Nucl. Med.* **10**: 103, 1969.

12. Pressman D. "Radiolabelled Antibodies." *Ann. N.Y. Acad. Sci.* **69**: 644, 1987.

13. Sfakianakis, G.N. and DeLand, F.H. "Radiommunodiagnosis and Radioimmunotherapy." *J. Nucl. Med.* **23**: 840, 1982.

14. Pressman, D., and Korngold, L., "The *in vivo* Localization of Anti-Wagner Osteogenic Sarcoma Antibodies." *Cancer* **6**: 619, 1953.

15. Korngold, L., and Pressman, D., "The Localization of Antilymphosarcoma Antibodies in the Murphy Lymphosarcoma of the Rat." *Cancer Res.* **14**: 96, 1954.

16. Order, S.E., Klein, J.L. and Ettinger, D. "Phase I-II Study of Radiolabelled Antibody Intergrated in the Treatment of Primary Hepatic Malignancies." *Int. J. Rad. Oncol. Biol. Phys.* **6**: 703, 1980.

17. Spar, I.L., Bale, W.F., Marrack, D., *et al.*, "I-131 Labelled Antibodies to Human

Fibrinogen : Diagnostic Studies and Therapeutic Trials." *Cancer* 20: 865, 1967.

18. Arnon, R., Bustin, M., Calef, E., *et al.*, "Immunological Crossreactivity of Antibodies to a Synthetic Undecapeptide Analogous to the Terminal Segment of CEA, with the Intact Protein and with Human Sera." *Proc. Natl. Acad. Sci.* 73: 2123, 197[illegible].

19. Damron, J.R., Beihn, R.M. and DeLand, F.H. "Detection o. Upper Abdominal Abscesses by Radionuclide Imaging." *Radiology* 120: 131, 1976.

20. Damron, J.R., Beihn, R.M., Selby, J.B., *et al.*, "Gallium Technetium Subtraction Scanning for the Localization of Subphrenic Abscesses." *Radiology* 113: 117, 1974.

21. Primus, F.J. and Goldenberg, D.M. "Immunological Considerations in the use of Goat Antibodies to Carcinoembryonic Antigen for the Radioimmunodetaction of Cancer." *Cancer Res.* 40: 2979, 1980.

22. Kohler, G., and Milstein, C. "Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity." *Nature* 256: 495, 1975.

23. Larson, S.M., Carrasquillo, J.A. and Reynolds, J.C. "Radioimmunodetection and Radioimmunotherapy." *Cancer Investigation* 2: 363, 1984.

24. Bullard, D.E. and Bigner, D.D. "Applications of Monoclonal Antibodies in the Diagnosis and Treatment of Primary Brain Tumors." *J. Neurosurg* 63: 2, 1985.

25. Goodwin, D.A. "Pharmacokinetics and Antibodies." *Journal of Nuclear Medicine* 28: 13[illegible], 1987.

26. Moldofsky P.J., Powe, J. and Hammond, N.D. "Tumor Detection Using Radiolabelled Monoclonal Antibodies." *CRC Critical Reviews in Clinical Laboratory Sciences* 25: 313, 1987.

27. Esteban, J.M., Colcher, D., Sugarbaker, P. *et al.*, "Quantitative and Qualitative Aspects of Radiolocalization in Colon Cancer Patients of Intravenously Administered MAb B72.3." *Int. J. Cancer* 39: 50, 1987.

28. Teh, J.G., Stacker, S.A., Thompson, C.H. *et al.*, "The Diagnosis of Human Tumours with Monoclonal Antibodies." *Cancer Surveys* 4: 149, 1985.

29. Leyden, M.J., Thompson, C.H., Lichtenstein, M. *et al.*, "Visualization of Metastases from Colon Carcinoma Using an Iodine-131 Radiolabeled Monoclonal Antibody." *Cancer* 57: 1135, 1986.

30. Gasparini, M., Ripamonti, M., Seregni, E. *et al.*, "Tumor Imaging of Colo-rectal Carcinoma with an Anti-CEA Monoclonal Antibody." *Int. J. Cancer* Supp. 2: 81, 1988.

31. Moldofsky, P., Powe, J., Mulhern, C.B., *et al.*, "Metastatic Colon Carcinoma Detected with Radiolabeled $F(ab')_2$ Monoclonal Antibody Fragments." *Radiology* 149: 349, 1983.

32. Sears, H.F., Mattis, J., Herlyn, D., *et al.*, "Phase I Clinical Trial of Monoclonal Antibody Treatment of Gastrointestinal Tumours." *Lancet* 1: 762, 1982.

33. 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.

34. DeLand, F.H., Kim, E.E., Primus, F.J., *et al.*, "*In Vivo* Radioimmunodetection of Occult Recurrent Colonic Carcinoma." *Am. J. Roentgenol.* 138: 145, 1982.

35. Fairweather, D.S., Bradwell, A.R., Dykes, P.W., *et al.*, "Improved Tumor Localization Using Indium-111 Labeled Antibodies." *Br. Med. J.* 287: 167, 1983.

36. Begent, R.H., Green, A.J., Bagshawe, K.D., *et al.*, "Liposomally Entrapped Second Antibody Improves Tumour Imaging with Radiolabelled (First) Antitumor Antibody." *Lancet* 2: 739, 1982.

37. Epenetos, A.A., Snook, D., Durbin, H., *et al.*, "Limitations of Radiolabeled Monoclonal Antibodies for Localization of Human Neoplasms." *Cancer Res.* 46: 3183, 1986.

38. Chatal, J.F., Saccavini, J.C., Famoleau, P., *et al.*, "Immunoscintigraphy of Colon Carcinomas." *J. Nucl. Med.* 311: 106, 1984.

39. Hnatowich, D.J., Griffin, T.W., Kosiuczyk, C., *et al.*, "Pharmacokinetics of an Indium-111 Labeled Monoclonal Antibody." *J. Nucl. Med.* 26: 253, 1983.

40. Henze, E., Kubel, R., Waitzinger, J., *et al.*, "Tumor Scintigraphy with I-131 Anti-CEA Monoclonal Antibodies and $F(ab')_2$ in Colorectal Cancer." Eur. J. Nucl. Med. 13: 125, 1987.

41. Epenetos, A.A., 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.

42. Jackson, P.C., Pitcher, E.M., Davies, J.O., *et al.*, "Radionuclide Imaging of Ovarian Tumours with a Radiolabelled (^{123}I) Monoclonal Antibody ($NDOG_2$)." *Eur. J. Nucl. Med.* 11: 22, 1985.

43. Ward, B.G., Mather, S.J., Hawkins, L.R., *et al.*, "Localization of Radioiodine Conjugated to the Monoclonal Antibody HMFG2 in Human Ovarian Carcinoma: Assessment of Intravenous and Intraperitoneal Routes of Administration." *Cancer Research* 47: 4719, 1987.

44. Javadpour, N., Kim, E.E., DeLand, F.H., *et al.*, "The Role of Immunodetection in the Management of Testicular Cancer." *J. Am. Med. Assoc.* 246: 45, 1981.

45. Babaian, R.J., Murray, J.L., Lamki, L.M., *et al.*, "Radioimmunological Imaging of Metastatic Prostatic Cancer with Indium-111-Labeled Monoclonal Antibody PAY 276." *The Journal of Urology* 137: 439, 1987.

46. Halpern, S.E., Hagan, P. Witztum, K.F., *et al.*, "Radioimmunoscintigraphy Using ^{111}In-labeled Monoclonal Antibodies and the Case for Immunotherapeutics." *J. Med. Tech.* 2: 301, 1985.

47. Murray, J.L., Rosenblum, M.G., Sobol, R.E., *et al.*, "Radioimmunoimaging in Malignant Melanoma with ^{111}In-labeled Monoclonal Antibody 96.5." *Cancer Res.* 45: 2376, 1985.

48. Rosenblum, M.G., Murray, J.L., Haynie, T.P., *et al.*, "Pharmacokinetics of ^{111}In-labeled Anti-p97 Monoclonal Antibody in Patients with Metastatic Malignant Melanoma." *Cancer Res.* 45: 2382, 1985.

49. Larson, S.M. "Radiolabeled Monoclonal Anti-Tumor Antibodies in Diagnosis and

Therapy." *J. Nucl. Med.* **26**: 538, 1985.

50. Murray, J.L., Lamki, L.M., Shanken, L.J., *et al* ., "Immunospecific Saturable Clearance Mechanisms for Indium-111-labelled Anti-Melanoma Monoclonal Antibody 96.5 in Humans." *Cancer Research* **48**: 4417, 1988.

51. Larson, S.M., Carrasquillo, J.A., McGriffin, R.W., *et al.*, "Use of I-131 Labeled, Murine Fab Against a High Molecular Weight Antigen of Human Melanoma : Preliminary Experience." *Radiology* **155**: 487, 1985.

52. Buraggi, G.L., Gallegaro, L., Turrin, A., *et al.*, "Immunoscintigraphy with I-123, Tc-99m and ^{111}In-labeled F(ab')$_2$ Fragments of Monoclonal Antibodies to a Human High Molecular Weight-Melanoma Associated Antigen." *J. Nucl. Med. Allied Sci.* **28**: 283, 1984.

53. Buraggi, G.L., Gallegaro, 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.

54. Carrasquillo, J.A., Abrams, P.G., Schroff, R.W., *et al.*, "Effect of Antibody Dose on the Imaging and Biodistribution of Indium-111 9.2.27 Anti-Melanoma Monoclonal Antibody." *J. Nucl. Med.* **29**: 39, 1988.

55. Taylor A., Milton, W., Eyre, H., *et al.*, "Radioimmunodetection of Human Melanoma with Indium-111-Labeled Monoclonal Antibody." *J. Nucl. Med.* **29**: 329, 1988.

56. Kirkwood, J.M., Neumann, R.D., Zoghbi, S.M., *et al.*, "Scintigraphic Detection of Metastatic Melanoma Using Indium 111/DTPA Conjugated Anti-gp240 Antibody (ZME-018)." *Journal of Clinical Oncology* **5**: 1247, 1987.

57. Rainsbury, R.M., Westwood, J.H., Coombes, R.C., *et al.*, "Location of Metastatic Breast Carcinoma by a Monoclonal Antibody Chelate Labelled with Indium-111." *Lancet* **1**: 934, 1983.

58. Symonds, E.M., Peerkins, A.C., Pimm, M.V., *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.

59. Williams, M.R., Perkins A.C., Campbell, F.C., *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.

60. Perkins, A.C. and Pimm, M.V. "Differences in Tumor and Normal Tissue Concentrations of Iodine- and Indium-Labelled Monoclonal Antibody." *Eur. J. Nucl. Med.* **11**: 295, 1985.

61. Powell, M.C., Perkins, A.C., Pimm, M.V., *et al.*, "Diagnostic Imaging of Gynecologic Tumors with the Monoclonal Antibody 791T/36." *Am. J. Obstet. Gynecol.* **157**: 28, 1987.

62. Sikora, K. "The Characterization of Glomas Using Human Monoclonal Antibodies: Mini-Review on Cancer Research." *Exp. Cell. Biol.* **52**: 189, 1984.

63. Bergmann J.F., Lumbroso, J.D., Manil, L., *et al.*, "Radiolabelled Monoclonal Antibodies Against Alpha-fetoprotein for *In Vivo* Localization of Human Hepatocellular Carcinoma by Immunotomoscintigraphy." *Eur. J. Nucl. Med.* **13**: 385, 1987.

64. Keenan A.M., Weinstein, J.N., Carrasquillo, J.A., *et al.*, "Immunolymphoscintigraphy and the Dose Dependence of In-111 Labeled T101 Monoclonal Antibody in Patients with Cutaneous T-cell Lymphoma." *Cancer Research* 47: 6093, 1987.

65. Carrasquillo, J.A., Mulshine, J.L., Bunn, P.A., *et al.*, "Indium-111 T101 Monoclonal Antibody is Superior to Iodine-131 T101 in Imaging of Cutaneous T-Cell Lymphoma." *J. Nucl. Med.* 28: 281, 1987.

66. Ghose, T., Blair, A.H., Martin, R.H., *et al.*, "Tumor Imaging by Antitumor Antibody-Linked Radionuclides: Experimental Models and Clinical Applications." in *Tumor imaging : The radiochemical detection of cancer.*" Burchiel, S.W. and Rhodes, B.A. (Eds). Masson Publishing Inc., USA, 1982, p.167.

67. Burchiel, S.W. "Expression and Detection of Human Tumor Associated Antigens." in *Radioimmunoimaging and radioimmunotherapy.* Burchiel, S.W. and Rhodes, B.A. (Eds). Elsevier Publishing Co. Inc., 1983, p.13.

68. McIntire, K.R. "Tumors markers: How useful are they?" *Hospital Practice* :55, 1984.

69. Zalcberg, J.R. and McKenzie, F.C. "Tumor-Associated Antigens - An Overview." *Journal of Clinical Oncology* 3: 876, 1985.

70. Feizi, T. "Carbohydrate Antigens in Human Cancer." *Cancer Syrveys* 4: 245, 1985.

71. Feizi T. "Monoclonal Antibodies Point to Carbohydrate Structures as Tumor-Associated Antigens." *Medical Biology* 61: 144, 1983.

72. Magnani, J.L. "Carbohydrate Differentiation and Cancer-Associated Antigens Detected by Monoclonal Antibodies." *Biochemical Society Transactions* 12: 543, 1984.

73. Ito, M., Suzuki, E., Naiki, M., *et al.*, "Carbohydrates as Antigenic Determinants of Tumor-Associated Antigens Recognized by Monoclonal Anti-Tumor Antibodies Produced in a Syngeneic Aystem." *Int. J. Cancer* 34: 689, 1984.

74. Friedenreich, V. "The Thomsen Haemagglutination Phenomenon." Levin and Munksgaard, Copenhagen, 1930.

75. Burnet, F.M., McCrea, J.F. and Stone, J.D. "Modification of Human Red Cells by Virus Action." *Br. J. Exp. Pathol.* 27: 228, 1946.

76. Springer, G.F. "Enzymatic and Nonenzymatic Alterations of Erythrocyte Antigen Surfaces." *Bacteriol. Rev.* 27: 191, 1963.

77. Springer, G.F., Desai, P.R., Murthy, M.S., *et al.*, "Precursors of the Blood Group MN Antigens as Human Carcinoma-Associated Antigens." *Transfusion* 19: 233, 1979.

78. Springer, G.F. and Desai, P.R. "Relation of Human Blood-Groups MN to Cancer Cell Surface Antigens and to Receptors for Oncogenic Viruses." *Annals of Clinical and Laboratory Science* 4: 294, 1974.

79. Vaith, P. and Uhlenbruck, G. "The Thomsen Agglutination Phenomenon: a Discovery Revisisted 50 Years Later." *Z. Immun. Forsch.* 154: 1, 1978.

80. Uhlenbruck, G. "The Thomsen-Friedenreich (TF) Receptor: An Old History with New Mystery." *Immunological Communications* 10: 251, 1981.

81. Howard, D.R. "Expression of T-Antigen on Polyagglutinable Erythrocytes and Carcinoma Cells: Preparation of T-Activated Erythrocytes, Anti-T Lectin, anti-T Absorbed Human Serum and Purified Anti-T Antibody." *Vox. Sang.* 37: 107, 1979.

82. Skutelsky, E., Lotan, R., Sharon, N., et al., "*Distribution of T-antigen on Erythroid Cell Surfaces: Studies with Peanut Agglutinin, an Anti-T Specific Lectin.*" *Biochim. Biophys. Acta.* 467: 165, 1977.

83. Landsteiner, K. and Levine, P. "On Individual Differences in Human Blood." *J. Exp. Med.* 47: 757, 1928.

84. Springer, G.F. "Blood Group T and Tn Antigens are Universal, Clonal, Epithelial Cell Adhesive, Autoimmunogenic Caracinoma Markers." in *Non-HLA antigens in health, aging and malignancy.* Alan R. Liss, Inc. N.Y., N.Y., 1983. p.157

85. Springer G.F. "T and Tn, General Carcinoma Autoantigens." *Science* 224: 1198, 1984.

86. Springer, G.F., Nagai, Y. and Tegtmeyer, H. "Isolation and Properties of Human Blood-Group NN and Meconium-Vg Antigens." *Biochemistry* 5: 3254, 1966.

87. Springer, G.F., Desai, P.R., Yang H.J., *et al.*, "Carcinoma-Associated Blood Group MN Precursor Antigens Against which All Humans Possess Antibodies." *Clin. Immunol. Immunopath.* 7: 426, 1977.

88. Kathan, R.H., Winzler, R.J. and Johnson, C.A. "Preparation of an Inhibitor of Viral Hemagglutination from Human Erythrocytes." *J. Exp. Med.* 113: 37, 1961.

89. Vainchenker, W., Vinci, G., Testa, U., *et al.*, "Presence of the Tn Antigen on Hematopoietic Progenitors from Patients with the Tn Syndrome." *Clin. Invest.* 75: 541, 1985.

90. Springer, G.F. and Desai, P.R. "Human Blood Group MN and Precursor Specificities: Structural and Biological Aspects." *Carb. Res. 40:* 183, 1975.

91. Fukuda, M.N., Fukuda, M. and Hakomori, S. "Cell Surface Modification by Endo-β-galactosidase." *J. Biol. Chem.* 254: 5458, 1979.

92. Springer, G.F. and Desai, P.R. "Tn epitopes, Immunoreactive with Ordinary Anti-Tn Antibodies, on Normal, Desialyated Human Erythrocytes and on Thomsen-Friedenreich Antigen Isolated Thereform." *Molecular Immunology* 22: 1303, 1985.

93. Orntoft, T. "A Method for T-antigen Demonstration by a Polyclonal Antibody and Peanut Lectin; Elimination of Cross-Reaction with Naturally Occuring Antibodies." *Virchows Arch.* 404: 159, 1984.

94. Kim, Y.S., Isaacs, R. and Perdomo, J. "Alterations of Membrane Glycoproteins in Human Colonic Adenocarcinoma." *Proc. Natl. Acad. Sci. USA.* 71: 4869, 1974.

95. Reitman, M., Trowbridge, I. and Kornfield, S. "Mouse Lymphoma Cell Lines Resistant to Pea Lectin and Defective in Fucose Metabolism." *J. Biol. Chem.* 255: 9900, 1980.

96. Kagawa, S., Takigawa, H., Ghazizadeh, M., *et al* ., "Immnunohistological Detection of T Antigen and ABH Blood Group Antigens in Upper Urinary Tract Tumours." *British Journal of Urology.* 57: 386, 1985.

97. Weinstein, R.S., Miller, A.W., and Coon, J.S. "Tissue Blood Group ABH and

Thomsen-Friedenreich antigens in Human Urinary Bladder Carcinoma." *Progress and Controversies in Oncological Urology.* p.249, 1984.

98. Springer, G.F., and Desai, P.R. "Interaction of Blood Group Mn-like Cancer Antigen and Human Cytotoxin." *Naturwis.* 61: 38, 1974.

99. Codington, J.F., Sanford, B.H. and Jeanloz, R.W. "Glycoprotein Coat of the TA3 Cell. Isolation and Partial Characterization of a Sialic Acid Containing Glycoprotein Fraction." *Biochemistry.* 11: 2559, 1972.

100. Noujaim, A.A., Shysh, a., Zabel, P., *et al* ., "Thomsen-Friedenreich Antigen. An Important Maker for the Radioimmunodetection of Cancer Using Macromolecular Probes." in *Radioimmunoimaging and radioimmunotherapy.* Burchiel, S.W. and Rhodes, R.A. (Eds) Elsevier Publishing Co. Inc., 1983. p.277.

101. Zabel, P.L., Noujaim, A.A., Shysh, A., *et al* ., "Radioiodinated Peanut Lectin: A Potential Radiopharmaceutical for Immunodetection of Carcinoma Expressing the T-antigen." *Eur. J. Med.* 8: 250, 1983.

102. Howard, S.P., Trenbath, T.P., Huggins, J.W., *et al* ., "Occurrence of Peanut Lectin Receptor (Blood Group T-antigen) in Ascites but not Solid and Cultured Forms of the 13762 Rat Mammary Adenocarcinoma." *J. Sup. Struct. (sup. 3)* p. 212, 1979.

103. Huggins, J.W., Trenbath, T.P. and Carraway, K.L. "Redistribution and Shedding of Concanavalin A and Peanut Lectin Receptor of Ascites Sublines of the 13762 Rat Mammary Adenocarcinoma." *J. Sub. Struct. (sup. 3)* p. 212, 1979.

104. Eu, S.M.. "Radioiodinated Peanut Lectin and Monoclonal Antibodies for the Detection of T-Antigen Expressing Tumors and Metastases." M.Sc. Thesis, University of Alberta, 1985.

105. Springer, G.F., Codington, J.F., and Jeanloz, R.W. "Surface Glycoprotein From a Mouse Tumor Cell as Specific Inhibitor of Antihuman Blood-Group Agglutinin." *J. N. C. I.* 49: 1469, 1972.

106. Springer, G.F., Desai, P.R., Robinson, M.K., *et al* ., "The Fundamental and Diagnostic Role of T and Tn Antigens in Breast Carcinoma at the Earliest Histological Stage and Throughout." *Tumor Markers and Their Significance in the Management of Breast Cancer.* p.47, 1986.

107. Springer, G.F., Murphy, S.M., Desai, P.R., *et al* ., "Patients' Immune Response to Breast and Lung Carcinoma-Associated Thomsen-Friedenreich (T) Specificity." *Klin. Wochenschr.* 60: 121, 1982.

108. Klein, P.J., Newman, R.A., Muller, P., *et al* ., "The Presence and Significance of the Thomsen-Friedenreich Antigen in Mammary Gland." *J. Cancer Res. Clin. Oncol.* 93: 205, 1979.

109. Limas, C., and Lange, P. "T-antigen in Normal and Neoplastic Urothelium." *Cancer.*58: 1236, 1986.

110. Summers, J.L., Coon, J.S., Ward, R.M., *et al* ., "Prognosis in Carcinoma of the Urinary Bladder Based Upon Tissue Blood Group ABH and Thomsen-Friedenreich Antigen Status and Karyotype of the Initial Tumor." *Cancer Research* 43: 934, 1983.

111. Vafier, J.A. Javadpour, N., Worsham, G.F., *et al* ., "Double Blind Comparison of

T-antigen and ABO(H) Cell Surface Antigens in Bladder Cancer." *Urology.* Vol.XXIII, No.4, 1984.

112. Yuan, M., Itzkowitz, S.H., Boland, C.R., *et al* ., "Comparison of T-antigen Expression in Normal, Premalignant, and Malignant Human Colonic Tissue Using Lectin and Antibody Immunohistochemistry." *Cancer Research.* **46**: 4841, 1986.

113. Orntoft, T.F., Mors, N.P., Erikson, G., *et al* ., "Comparative Immunoperoxidase Demonstration of T-antigens in Human Colorectal Carcinomas and Morphologically Abnormal Mucosa." *Cancer Research* **45**: 447, 1985.

114. Springer, G.F., Desai, P.R., and Banatwala, I. "Blood Group MN Specific Substances and Precursors in Normal and Malignant Human Breast Tissues." *Naturwissenschaften.* **61**: 457, 1974.

115. Springer, G.F., and Desai, P.R. "Cross-Reacting Carcinoma Associated Antigens with Blood Group and Precursor Specificities." *Transplant Proc.* **9**: 1105, 1977.

116. Springer, G.F., Desai, P.R., and Banatwala, E.I. "Blood Group MN Antigens and Precursors in Normal and Malignant Breast Glandular Tissue." *J. N. C. I.* **54**: 335, 1975.

117. Springer, G.F., and Tegtmeyer, H. "Origin of Anti-Thomsen-Friedenreich (T) and Tn Agglutinins in Man and in White Longhorn Chicks." *Br. J. Haematol.* **47**: 453, 1981.

118. Stillmark, H. "Arbeiten des pharmakologischen Institutes zu Dorpat." R. Kobert ed. 3: 57, 1889.

119. Elfstrand, M. "Gorbersdorfer Veroffentlichungen". R. Korbet, Ed., pp 1-159 Enke, Stuttart.

120. Boyd, W.C. and Shapleigh, E. "Specific Precipitating Activity of Plant Agglutinins (Lectins)." *Science* **119**, 419, 1954.

121. Gold, E.R. and Phelps, C.F. "Provisional Classification of Receptor-Specific Proteins (Antibody-Like Substances)." *Z. Immuntiaetsforsch., Exp. Klin. Immunol.* **143**: 430, 1972.

122. Sharon, N. and Lis, H. "Lectins: Cell Agglutinating and Sugar Specific Proteins." *Science* **177**: 949, 1972.

123. Goldstein, I.J., Hughes, R.G., Monsigny, M., *et al.*, "What Should Be Called a Lectin?" *Nature* **285**: 66, 1980.

124. Kocourek, J. and Horejsi, V. "Defining a Lectin." *Nature* **290**: 188, 1981.

125. Makela, O. and Makela, P. "Some New Blood Group Specific Phytagglutinins." *Ann. Med. Exp. Biol. Fenn.* 34402, 1957.

126. Lis, H. and Sharon, N. "Soy Bean (Glycine max) Agglutinin." *Methods in Enzymology.* V. Ginsburg ed. **28B**: 360, 1973.

127. Horejsi, V. and Kocourek, J. "Studies on Phytohemagglutinins. XVIII. Affinity Electrophoresis of Phytohemagglutinins." *Biochim. Biophys. Acta.* **336**: 338, 1974.

128. Pongor, S. and Riedl, Z. "A Latex Agglutination Test for Lectin Binding." *Anal Biochem.* **129**:51, 1983.

129. Kint, J. "A Solid Phase Procedure For Dectecting and Evaluating Lectins." *Arch. Int. Physiol. Biochim.* **90**: B41, 1982.

130. Lis, H. and Sharon, N. "Affinity Chromatography for the Purification of Lectins." *J. Chromatogr.* **215**: 361, 1981.

131. Porath, J. and Kristiansen, T. *The Proteins* H. Neurath, R. Hill and C. Boeder, eds. **1**: 95, 1975.

132. Pazur, J. "Affinity Chromatography of Macromolecular Substances on Adsorbents Bearing Carbohydrate Ligands." *Adv. Carbohydr. Chem. Biochem.* **29**: 405, 1981.

133. Goldstein, I.J. and Hayes, C.E. "The lectins: Carbohydrate Binding Proteins of Plants and Animals." *Adv. Carbo. Chem. Biochem.* **35**: 127, 1978.

134. Rudiger, H. "Lectins- An Introduction." in *Lectins:biology, biochemistry, clinical biochemistry. vol I* Bog-Hansen, T.C. and Spengler, G.A. (Eds) Walter de Gruyter. 1981. p. 3

135. Lis, H. and Sharon, N. "Lectins: Their Chemistry and Application to Immunology." in *The Antigens* Vol. 4, Sela, M. (Ed) Academic Press, New York, 1977. p. 429.

136. Landsteiner, K. *The Specificity of Seriological Reaction.* Rev. ed. 1962.

137. Goldstein, I.J. and Poretz, R.D. "Isolation, Physicochemical Characterization, and Carbohydrate-Binding Specificity of Lectins." in *The Lectins: Properties, Functions, and Applications in Biology and Medicine* Liener, I.E., Sharon, N., Goldstein, I.J. (Ed), Academic Press, Florida, 1986.

138. Goldstein I.J., Iyer R.N., Smith E.E., *et al* "Protein-Carbohydrates Interaction. XX. The Interaction of Concanavalin A with Sophorose and Some of Its Derivates." *Biochemistry* **6**: 2373, 1967.

139. Van Wauwe J.P., Loonteins F.G. and DeBruyne C.K. "Carbohydrate Binding Specificity of the Lectin from the Pea (*Pisum Sativum*)." *Biochim. Biophys. Acta* **379**: 456, 1975.

140. Allen A.K., Desai N.N. and Neuberger, A. "The Purification of the Glycoprotein Lectin from the Broad Bean (*Vicia faba*) and a Comparsion of its Properties with Lectins of Similar Specificity." *Biochem. J.* **155**, 127, 1976.

141. Roberts, D. and Goldstein, I. "Hydrophobic Binding Properties of the Lectin from Lima Beans (*Phaseolus lunatus*)." *J. biol. Chem.* **257**: 11274, 1982.

142. Roberts, D. and Goldstein, I. "Binding of Hydrophobic Ligands to Plant Lectins: Titration with Arylaminoaphthalenesulfonates." *Arch. Biochem. Biophys.* **224**: 479, 1983.

143. Hardman, K. and Ainsworth, C. "Structure of the Concanavalin A-Methyl α-D-Mannopyranoside Complex at 6-A Resolution." *Biochemistry.* **15**: 1120, 1976.

144. Lemieux, R. *Frontiers of Chemistry.* K. Laidler (Ed) p. 3

145. Hindsgaul, O., Norberg, T., Lependu, J., *et al.*, "Synthesis of Type 2 Human Blood Group Antigenic Determinants. The H, X and Y Haptens and Variations of the H Type 2 Determinant as Probes for the Combining Sites of the Lectin I of Ulex europaeus." *Carbohydr. Res.* **109**: 109,1982.

146. Baker, D., Sugii, S., Kabat, E.. *et al.*, "Immunochemical Studies on the Combining Sites of Frossman Hapten Reactive Hemagglutinins from *Dolichos biflorus, Helix pomatia,* and *Wistaria floribunda.*" *Biochemistry.* 22: 2741, 1983.

147. Roberts, D. and Goldstein, I. "Effect of Carbohydrate and Metal Ion Binding on the Reactivity of the Essential Thiol Groups of Lima Bean lectin." *J. Biol. Chem.* 259: 903, 1984.

148. Kronis, K. and Carner, J. "Thermodynamics of Wheat Germ Agglutinin Sialyloligosaccharide Interaction by Proton Nuclear Magnetic Resonance." *Biochemistry.* 24: 834, 1985.

149. Barondes S.H. "Lectins: Their Multiple Endogenous Cellular Functions". *Ann. Rev. Biochem.* 50: 207, 1981.

150. Nicolson, G. "Transmembrane Control of the Receptors on Normal and Tumor Cells." *Biochim. Biophys. Acta.* 457: 57, 1976.

151. Sharon, N. and Lis, H. "Use of Lectins for the Study of Membranes." *Methods Membr. Biol.* 3: 147, 1975.

152. Lis, H. and Sharon, N. In *The biochemistry of plants* Vol. 6 Marcus, A. (Ed) Academic Press, New York, 1981. p.371.

153. Nowell, P.C. "Phytohemagglutinin: An Inhibitor of Mitosis in Cultures of Normal Human Leukocytes." *Cancer Res.* 20: 462, 1960.

154. Hume, D. and Wiedemann, M. "Mitogenic Lymphocyte Transformation." Elsevier/North-Holland Biomedical Press, Amsterdam, 1980.

155. Cohen, S., Pick, E., and Oppenheim, J. (Eds) "Biology of the Lymphokines." Academic Press, New York, 1979.

156. Klaus,G. and Hawrylowicz, C. "Cell Cycle Control in Lymphocyte Stimulation." *Immunol. Today.* 5: 15, 1984.

157. Brown, J.C. and Hunt, R.C. "Lectins." *Int. Rev. Cytol.* 52: 277, 1978.

158. Fodstad, O. and Pihl, A. "Effect of Ricin and Abrin on Survival of L1210 Leukemic Mice and on Leukemic and Normal Bone Marrow Cells." *Int. J. Cancer* 22: 558, 1978.

159. Itaya, K., Itoh, M., Tanaka, I., *et al.*, "Tumor-Suppressing Effect of a Monovalent Monomeric Derivative of Concanavalin A Obtained by Photoalkylation with Chloroacetamide." *Biomed. Res.* 2: 501, 1981.

160. Maddox D.E., Goldstein I.J. and LoBuglio A.F. "*Griffonia simplicifolia* I Lectin Mediates Macrophage-Induced Cytotoxicity Against Ehrlich Ascites Tumor." *Cell. Immunol.* 71: 202, 1982.

161. Bird, G.W.G. "Anti-T in peanuts." *Vox Sang.* 9: 748, 1964.

162. Salunke, D.M., Islam Khan, M., Surolia, A., *et al* ., "Crystallization and Preliminary X-ray Studies of the Anti-T Lectin from Peanut (*Arachis hypogaea*)." *J. Mol. Biol.* 154: 177, 1982.

163. Hakomori S. "Glycospingolipids in Cellular Interaction, Differantiation and

Oncogenesis." *Ann. Rev. Biochem.* 50: 733, 1981.

164. Nakahara K., Ohashi T., Oda T., *et al.*, "Asialo GM_1, as well as a Cell-Surface Marker Detected in Acute Lymphoblastic Leukemia". *New Engl. J. Med.* 302: 674, 1980.

165. Terao, T., Irimura, T. and Osawa, T. "Purification and Characterization of a Hemagglutinin from Arachis hypogaea." *Hoppe-Seylor's Z. Physiol. Chem.* 356: 1685, 1975.

166. Lotan, R., Skutelsky, E., Danon, D., *et al* ., "The Purification, Composition, and Specificity of the Anti-T Lectin from Peanut (Arachis hypogaea)." *Journal of Biological Chemistry* 250: 8518, 1975.

167. Pujol, F. and Cesari, I. "A Simplified Methodology for Purification of Peanut (Arachis hypogaea) Agglutinin." *Journal of Biochemical and Biophysical Methods* 13: 131, 1986.

168. Merry A.H., Rawinson V.I. and Stratton F. "A Rapid Purification Method for Peanut Anti-T Lectin." *Immunol. Commun.* 10: 265, 1981.

169. Fish W.W., Hamlin L.M. and Miller R.L. "The Marcomolecular Properties of Peanut Agglutinin." *Arch. Biochem.* 190(2): 693, 1978.

170. Peuppke, S.G. "Multiple Molecular Forms of Peanut Lectin: Classification of Isolectins and Isolectin Distribution Among Genotypes of the Genus *Arachis*." *Carbo. Res.* 51: 107, 1976.

171. Salunke, D., Swamy, M., Khan, M., *et al* ., "Arrangement of Subunits in Peanut Lectin." *Journal of Biological Chemistry* 260: 13576, 1985.

172. Salunke, D., Khan, M., and Surolia, A. "Preparation and Preliminary X-ray Studies of Three Acidic pH Crystals Forms of the Anti-T Lectin from Peanut (*Arachis hypogaea*)." *FEBS Lett.* 156: 127, 1983.

173. Herrmann, M., Richardson, C., Setzler, L., *et al.*, "A Circular Dichroic Investigation of the Secondary Structure of Lectins." *Biopolymers.* 17: 2107, 1978.

174. Neurohr, K.J., Bundle, D.R., Young, N.M., *et al* ., "Binding of Dissaccharides by Peanut Agglutinin as Studied by Ultraviolet Difference Spectroscop[illegible]." *Eur. J. Biochem.* 123: 305, 1982.

175. Neurohr, K.J., Young, N.M., and Mantsch, H.H. "Determination of the Carbohydrate-Binding Properties of Peanut Agglutinin by Ultraviolet Difference Spectroscopy." *J. Biol. Chem.* 255: 9205, 1980.

176. Decastel, M., Bourrillon, R. and Frenay, J.R. "Cryoinsolubility of Peanut Agglutinin. Effects of Saccharides and Neutral Salts." *J. Biol. Chem.* 256: 9003, 1981.

177. Newman, R.A. "Heterogeneity Among the Anti-Tf Lectins Derived from *Arachis Hypogaea*." *Hoppe Seylers Z. Physiol. Chem.* 358: 1517, 1977.

178. Novogrodsky, A., Lotan, R., Ravid, A., *et al.*, "Peanut Agglutinin a New Mitogen that Binds to Galactosyl Sites Exposed After Neuraminidase Treatment." *J. Immunol.* 115: 1243, 1975.

179. Uhlenbruck, G., and Gielen, W. "Serologische Eigenschaften von Ovalbumin nach Kupplung mit diazotiertem Amino-phenyl-N-acetyl-β-galaktosaminid : Ein Beitrag zur

Spezifitat von Anti-A*hel Hoppe Seyler's Z. Physiol. Chem.* **348**: 1693, 1967.

180. Kaifu, R. and Osawa, T. "Synthesis of *O*-β-D-Galactopyranosyl -(1→3)-O(-2-acetamido-2-deoxy-alpha(-β-)-D-galactopyranosyl) -N-tosyl-L-serine and their Interaction with D-Galactose-Binding Lectins." *Carbohydr. Res.* **69**: 79, 1979.

181. Pereira, M.E.A. and Kabat, E.A. "Immunochemical Studies on Blood Groups LXII. Fractionation of Hog and Human A, H, and AH Blood Group Active Substance on Insoluble Immunoadsorbents of *Dolichos* and *Lotus* Lectins." *J. Exp. Med.* **143**: 422, 1976.

182. Ohanessian, J., and Caron, M. "A Spectrophotometric Study of the Carbohydrate Binding Site of Peanut Lectin." in *Lectins: Biology, clinical biochemistry, vol. 3.* Bog-Hansen, T.C. and Spengler, G.A. (Eds), Walter de Gruyter, Berlin, 1983.

183. Jimbo, A. and Matsumoto, I. "Involvement of a Tyrosyl Residue in the Interaction of Peanut Lectin with Lactose." *J. Biochem. (Tokyo)* **91**: 945, 1982.

184. Neurohr, K.J., Young, N.M., Smith, I.C.P., *et al.*, "Kinetics of Binding of Methyl α- and β-D-Galactopyranoside to Peanut Agglutinin: A Carbon-13 Nuclear Magnetic Resonance Study." *Biochemistry* **20**: 3499, 1981.

185. Neurohr, K.J., Mantsch, H.H., Young, N.M., *et al.*, "Carbon-13 Nuclear Magnetic Resonance Studies on Lectin-Carbohydrate Interactions: Binding of Specifically Carbon-13 Labeled Methyl β-D-Lactoside to Peanut Agglutinin." *Biochemistry* **21**: 498, 1982.

186. Cooper, H.S. "Lectins as Probes in Histochemistry and Immunohistochemistry." *Human Pathol.*, **15**: 904, 1984.

187. Glockner, W.M., Newman, R.A., Dahr, W., *et al* ., "Alkali-Labile Oligosaccharide from Glycoproteins of Different Erythrocytes and Milk Fat Globule Membranes." *Biochem. Biophys. Acta.* **443**: 402, 1976.

188. Rosenberg, M., Chitayat, D., Tzehoval, E., *et al.*,"Detection and Enumeration of Monocytes in Human Blood with Peanut A [illegible]tinin." *Journal of Immunological Methods* **81**: 7, 1985.

189. Uhlenbruck, G., Pardoe, G.I. and Bird, G.W.G. "On the Specificity of Lectins with a Board Agglutination Spectrum : II. Studies on the Nature of the T-antigen and the Specific Receptors for the Lectin of *Arachis Hypogaea* (Ground Nut)." *Z. Immunitaetsforsch.* **138**: 423, 1969.

190. McConnell, I., Munroe, A., and Waldmann, H. "The Immune System.", 2nd Ed. Blackwell, Oxford, 1981.

191. London. J., Berrih, S., and Bach, J. "Peanut Agglutinin I. A New Tool for Studying T Lymphocyte Subpopulations." *J. Immunol.* **121**: 438, 1978.

192. Nakamura, T., Tanimoto, K., Nakano, K., *et al.*, "Isolation of Human Supressor T-Cells by Peanut Agglutinin." *Int. Archs. Allery Appl. Immun.*, **68**: 338, 1982.

193. Reisner, Y., Biniaminov, M., Rosenthal, E., *et al.*, "Interaction of Peanut Agglutinin with Normal Human Lymphocytes and with Leukemic Cells." *Proc. Nat. Acad. Sci. USA.*, **76**: 447, 1979.

194. Rose, M.L. and Malchiodi, F. "Binding of Peanut Lectin to Thymic Cortex and Germinal Centres of Lymphoid Tissue." *Immunol.* **42**: 583, 1981.

195. Ballet, J.J., Fellous, M., Sharon, N., *et al.*, "Reactivity of Human Lymphoid and Lymphoblastoid Cells with Peanut Agglutinin: Detection of a Blood Cell Subset Which Lacks Detectable Membrane HLA." *Scand. J. Immunol.* **11**: 555, 1980.

196. Levin, S., Russell, E.C. Blanchard, D., *et al.*, "Receptors for Peanut Agglutinin (*Arachis Hypogaea*) in Childhood Acute Lymphoblastic Leukemia: Possible Clinical Significance." *Blood.* **55**: 37, 1980.

197. Veerman, A.J.P., Hogeman, P.H.G., Huismans, D.R., *et al.*, "Peanut Agglutinin, A Marker of T-Cell Acute Lymphoblastic Leukemic with a Good Prognosis." *Cancer Res.* **45**: 1890, 1985.

198. Glockner, W.M., Kaulen, H.D. and Uhlenbruck, G. "Immunochemical Determination of the Thomsen- Friedenreich Antigen (T-antigen) on Platelet Plasma Membranes." *Thrombos. Haemostas.*, **39**: 186, 1978.

199. Coapman, R., and Cooper, H. "Peanut Lectin Binding Sites in Human Fetal Colon." *Arch. Pathol. Lab. Med.* **110**: 124, 1986.

200. Johnson, L., and Hageman, G. "Enzymatic Characterization of Peanut Agglutinin-binding Components in the Retinal Interphotoreceptor Matrix." *Exp. Eye Res.* **44**: 553, 1987.

201. Hageman, G., and Johnson, L. "Biochemical Characterization of the Major Peanut-Agglutinin-Binding Glycoproteins in Vertebrate Retinae." *Journal of Comparative Neurology* **249**: 499, 1986.

202. Barresi, G., Tuccari, G., and Arena, F. "Peanut Lectin Binding to the Alveolar Lining Layer in Hyaline Membrane Disease." *Virchows Arch A* **411**: 157, 1987.

203. Viale, G., Dell'Orto, P., Colombi, R., *et al.*, "Lectin Binding Sites on Semithin Sections of Epoxy-Embedded Tissues." in *Lectins- Biology, Biochemistry, Clinical Biochemistry* Vol 3., Boghansen, T.C., Splenger, G.A., (eds.), Walter de Gruyter, 1983. pp 199.

204. Holthofer, H. "Lectin Binding Sites in Kidneys: A Comparative Study of 14 Animal Species." *J. Histochem. Cytochem.* **31**: 531, 1981.

205. Holthofer, H., Miettinen, A., *et al.*, "Comparison of Lectin Binding Sites in the Kidneys of Different Animal Species." in *Lectins- Biology, Biochemistry, Clinical Biochemistry* Vol 3., Boghansen, T.C., Splenger, G.A., (eds.), Walter de Gruyter, 1983. pp 199.

206. Schulte, B.A. and Spicer, S.S. "Histochemicsl Evaluation of Mouse and Rat Kidneys with Lectin-Horseradish Peroxidase Conjugates." *Am. J. Anatomy* **168**: 345, 1983.

207. Vierbuchen, M., Klein, P.J., Uhlenbruck, G., *et al.*, "The Significance of Lectins Receptors in the Kidney and in Hypernephroma (Renal Adenocarcinoma)." in *Recent Results in Cancer Research, Cancer Chemotherapy and Immunopharmacology.* Mathe, G., Muggia, F.M. (Eds.), Springer-Verlag, N.Y., 1983, pp 68.

208. Raedler, A., Boehle, A., Otto, U., *et al.*, "Differences of Glycoconjugates Exposed on Hypernephroma and Normal Kidney Cells." *J. Urol.* **128**: 1109, 1982.

209. Boniface, G.R. "The Renal Excretion of Radioiodinated Peanut Lectin." Ph.D. Thesis,

University of Alberta, 1986.

210. Newman, R., Klein, P., and Rudland, P. "Binding of Peanut Lectin to Breast Epothelium, Human Carcinomas, and a Cultured Rat Mammary Stem Cell: Use of the Lectin as a Marker of Mammary Differentiation." *J. N. C. I.* 63: 1339, 1979.

211. Howard, D. and Batsakis, J. "Cytostructural Localization of a TumorAssociated Antigen." *Science* 210: 201, 1980.

212. Howard, D., Ferguson, P. and Batsakis, J. "Carcinoma-Associated Cytostructural Antigenic Alterations: Detection by Lectin Binding." *Cancer* 47: 2872, 1981.

213. Hageman, P., Bobrow, L., Van der Valk, M., *et al.*, "Reactions with Lectins on Benign and Malignant Lesions of the Human Breast." *Lectins* 3: 105, 1983.

214. Calafat, J., Hageman, P. and Janssen, H. "Ultrastructural Localization of Peanut Agglutinin Receptors on Cells of Human Mammary Tumors." *Lectins* 3: 131, 1983.

215. Vierbuchen, M., Klein, P., Rosel, S., *et al.*, "Endocrine Therapeutical Studies on Carcinoma-Induced Rat Mammary Carcinomas Characterized by Hormone-Dependent Lectin-Binding Sites." *Lectins* 3: 145, 1983.

216. Klein, M., Vierbuchen, M., Fischer, J., *et al.*, "The Characterization of Milk Proteins by Lectins and Antibodies in Hormone Dependent Breast Cancer." *Lectins* 3: 157, 1983.

217. Klein, P., Vierbuchen, M., Fischer, J., *et al.*, "The Significance of Lectin Receptors for the Evaluation of Hormone Dependence in Breast Cancer." *J. Steroid Biochem.* 19: 839, 1983.

218. Seitz, R., Fischer, K., Stegner, H., *et al.*, "Detection of Metastatic Breast Carcinoma Cells by Immunofluorescent Demonstration of Thomsen-Friedenreich Antigen." *Cancer* 54: 830, 1984.

219. Bocker, W., Klaubert, A., Bahnsen, J., *et al.*, "Peanut Lectin Histochemistry of 120 Mammary Carcinomas and its Relation to Tumor Type, Grading, Staging, and Receptor Status." *Virchows Arch.* 403: 149, 1984.

220. Walker, R., Hawkins, R. and Miller, W. "Lectin Binding and Steroid Receptors in Human Breast Carcinomas." *Journal of Pathology* 147: 103, 1985.

221. Helle, M. and Krohn, K. "Reactivity of a Monoclonal Antibody Recogizing an Estrogen Receptor Regulated Glycoprotein in relation to Lectin Histochemistry in Breast Cancer." *Virchows Arch.* 410: 23, 1986.

222. Boland, C., Montgomery, C. and Kim, Y. "Alterations in Human Colonic Mucin occuring with Cellular Differentiation and Malignant Transformation." *Proc. Natl. Acad. Sci. USA* 79: 2051, 1982.

223. Cooper, H. "Peanut Lectin-Binding Sites in Large Bowel Carcinoma." *Laboratory Investigations* 47: 383, 1982.

224. Fischer, J., Klein, P., Vierbuchen, M., *et al.*, "Histochemical and Biochemical Characterization of Glycoprotein Components in Normal Gastric Mucosa, Intestinal Metaplasia and Gastric Cancers with Lectins." *Lectins* 3: 167, 1983.

225. Cooper, H. and Reuter, V. "Peanut Lectin-Binding Sites in Polyps of the Colon and

Rectum." *Laboratory Investigations* 49: 655, 1983.

226. Picard, J. and Feizi, T. "Peanut Lectin and Anti-Ii Antibodies Reveal Structural Differences among Human Gastrointestinal Glycoproteins." *Molecular Immunology* 20: 1215, 1983.

227. Lehman, T., Cooper, H. and Mulholland, S. "Peanut Lectin Binding Sites in Transitional Cell Carcinoma of the Urinary Bladder." *Cancer* 53: 272, 1984.

228. MaCartney, J. "Lectin Histochemistry of Galactose and *N*-AcetylGalactosamine Glycoconjugates in Normal Gastric Mucosa and Gastric Cancer and the Relationship with ABO and Secretor Status." *Journal of Pathology* 150: 135, 1986.

229. Alroy, J., Szoka, F.C., Heaney, J.A., *et al.*, "Lectins as a Probe for Carbohydrate Residues in Non-Neoplastic Urothelium of Human Urinary Bladder." *J. Urology*, 128: 189, 1982.

230. Coon, J.S., Weinstein, R.S. and Summers J.L. "Blood Group Precursor T-Antigen Expression in Human Urinary Bladder Carcinoma." *Am. J. Clin. Pathol.*, 77: 692, 1982.

231. Ghazizadeg, M., Kagawa, S. Izumi, K., *et al.*, "Immunohistochemical Localization of T-Antigen Like Substance in Benign Hyperplasia and Adenocarcinoma of the Prostate." *J. Urology*, 132: 1127, 1984.

232. Muramatsu, T. Gachelin, G., Damonville, M., *et al.*, "Cell Surface carbohydrates of Embryonal Carcinoma Cells: Polysaccharide Side Chains of F9 Antigens and of Receptors to Two Lectins. FBP and PNA." *Cell* 18: 183, 1979.

233. Bychkov, V. and Toto, P.D. "Wheat Germ and Peanut Agglutinin Binding to Normal, Dysplactic and Neoplastic Cervical Epithelium." *Gynecol. Obstet. Invest.* 21: 158, 1986.

234. Ree, H.J. and Kadin M.E. "Peanut Agglutinin : A Useful Marker for Histocytosos X and Interdigitating Reticulum Cells." *Cancer* 57: 282, 1986.

235. Hajdu, I., Zhang, W. and Gordon, G.B. "Peanut Agglutinin Binding as a Histochemical Tool for Diagnosis of Eosinophilic Granuloma." *Arch. Pathol. Lab. Med.* 110: 719, 1986.

236. Shysh, A., Eu, S.M., Noujaim, A.A., *et al.*, "In Vivo Localization of Rdaioiodinated Peanut Lectin in a Murine TA_3/Ha Mammary Carcinoma Model." *Eur. J. Nucl. Med.* 10: 68, 1985.

237. Shysh, A., Eu, S.M., Noujaim, A.A., *et al.*, "Radioimmunodetection of Murine Mammary Adenocarcinoma (TA_3/Ha) Lung and Liver Metastases with Radioiodinated PNA." *Int. J. Cancer*, 35: 113, 1985.

238. Yokoyama, K., Aburano, T., Watanabe, N., *et al.*, "Tumor Affinity of Radiolabeled Peanut Agglutinin Compared with that of Ga-67 Citrate in Animal Models." *J. Nucl. Med.*, 26: P111, 1985. (abst).

239. Holt, S., Wilkinson, A., Suresh, M.R. *et al.*, "Radiolabelled Peanut Lectin for the Scintigraphic Detection of Cancer." *Cancer Letter* 25: 55, 1984.

240. Abdi, E.A., Kamitomo, V.J., McPherson, T.A., *et al.*, "radioiodinated Peanut Lectin: Clinical Use as a Tumoyr-Imaging Agent and Potential Use in assessing Renal-Tubular Function." *Eur. J. Nucl. Med.*, 11: 350, 1986.

241. Baldwin, R.M. "Chemistry of Radioiodine." *Appl. Radiat. Isot.* 37: 817, 1986.

242. Bolton, A.E.. *Radioiodination Techniques.* Amersham Radiochemical Centre Puntarium Ltd. Middlesex, 1977.

243. Turner, C. "A Comparative Study of Radiochelated and Radioiodinated Monoclonal Antibodies." M.Sc. Thesis, University of Alberta, 1987.

244. Fraker, P.J. and Speck, J.C. "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.

245. Gelewitz, E.W., Riedemann W.L. and Klotz, I.M. "Some Quantitative Aspects of the Reaction of Diazonium Compounds with Serum Albumin." *Arch. Biochem. Biophys.* 53: 411, 1954.

246. Abram, S., Abram, U., Spies H., *et al.*, "In-113m Chelate Complexes - I. *In Vitro* Investigations on a Series of Alkylvaried Dithiocarbamato Complexes with In-113m." *Int. J. Appl. Radiat. Isot.* 36: 653, 1985.

247. Lanteigne, D. and Hnatowich, D.J. "The Labelling of DTPA-coupled Proteins with Tc-99m." *Int. J. Appl. Radiat. Isot.* 35: 617, 1984.

248. Childs, R.L. ang Hnatowich, D.J. "Optimum Conditions for Labeling of DTPA-Coupled Antibodies with Technetium-99m." *J. Nucl. Med.* 26: 293, 1985.

249. Goodwin, D.A., Sundberg, M.W., Diamanti, C.I., *et al.*, "Indium-111 Radiopharmaceutivals in Cancer Localization." in *Radiologic and other Biophysical Methods of Tumor Diagnosis* 18th Annual Conference on Cancer (Proceedings). Yearbook Med. Pub. Inc. Chicago. 1973. pp57-88.

250. Sundberg, M.W., Meares, C.F., Goodwin, D.A., *et al.*, "Chelating Agents for the Binding of Metal Ions to Marcomolecules." *Nature* 250: 587, 1974.

251. Sundberg, M.W., Meares, C.F., Goodwin, D.A., *et al.*, "Selective Binding of Metal Ions to Marcomolecules Using Bifunctional Analogs of EDTA." *J. Med. Chem.* 17: 1304, 1974.

252. Gokce, A., Nakamura, R.M., Tubis, M., *et al.*, "Synthesis of Indium-Labeled Antibody-Chelate Conjugate for Radioassays." *Int. J. Nucl. Med. Biol.* 9: 85, 1982.

253. Pritchard, J.H., 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.

254. 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.

255. Meares, C.F., Goodwin, D.A., Leung, C.S., *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.

256. Krejcarek, G.E., and Tucker K.L. "Covalent Attachment of Chelating Groups to Macromolecules." *Biochem. Biophys. Res. Commun.* 77: 581, 1976.

257. Scheinberg, D.A., Strand, M., and Gansow, O.A. "Tumor Imaging with Radioactive Metal Chelates Conjugated to Monoclonal Antibodies." *Science* 215: 1511, 1982.

258. Yeh, S.M., Sherman, D.G. and Meraes, C.F. "A New Route to 'Bifunctional' Chelating Agents : Conversion of Amino Acids to Analogs of Ethylenedinitrilotetraacetic Acid." *Analytical Biochemistry* 100: 152, 1979.

259. Meares, C.F., McMall, M.J., Reardan, D.T., *et al.*, "Conjugation of Antibodies with Bifunctional Chelating Agents : Isothiocyanate and Bromoacetamide Reagents, Methods of Analysis, and Subaequent Addition of Metal Ions." *Analytical Biochemistry* 142: 68, 1984.

260. Meares, C.F. "Chelating Agents for the Binding of Metal ions to Antibodies." *Nucl. Med. Biol.* 13: 311, 1896.

261. Goodwin, D.A., Meares, C.F., McCall, M.J., *et al.*, "Chelate Conjugates of Monoclonal Antibodies for Imaging Lymphoid Structures in the Mouse." *J. Nucl. Med.* 26: 493, 1983.

262. Green, M.A., Welch, M.J., Mathias, C.J., *et al.*, "Evaluation of PLED as a Chelating Ligand for the Preparation of Gallium and Indium Radiopharmaceuticals." *Int. J. Nucl. Med. Biol.* 12: 381, 1985.

263. Haner, M., Eidson, A.F., Darnall, D.W., *et al.*, "Synthesis of Affinity-Label Chelates : A Noval Synthetic Method of Coupling Ethylenediaminetetraacetic cid to Amine Functional Groups." *Archives of Biochemistry and Biophysics* 231: 477, 1984.

264. Paik, C.H., Murphy, P.R., Eclelman, W.C., *et al.*, "Ottimization of the DTPA Mixed-Anhydride Reaction with Antibodies at Low Concentration." *J. Nucl. Med.* 24: 932, 1983.

265. Eckelman, W.C. and Paik, C.H. "Comparison of Tc-99m and In-111 Labeling of Conjugated Antibodies." *Nucl. Med. Biol.* 13: 335, 1986.

266. Hnatowich, D.J., Childs, R.L., Lanteigne, D., *et al.*, "The Preparation of DTPA-Coupled Antibodies Radiolabeled with Metallic Radionuclides : an Improved Method." *Journal of Immunological Methods* 65: 147, 1983.

267. Hnatowich, D.J., Layne, W.W., and Childs, R.L. "The preparation and Labeling of DTPA-Coupled Albumin." *Int. J. Rad. Appl. Isot.* 33: 327, 1982.

268. Hnatowich, D.J. and McGann, J. "DTPA-Coupled Proteins - Procedures and Precautions." *Nucl. Med. Biol.* 14: 563, 1987.

269. Wang, T.S.T., Srivastava, S.C., Fawwaz, R.A., *et al.*, "Preparation of In-111 Labeled Monoclonal Antibody (MoAb) to High-Molecular-Weight Melanoma Associated Antigen (HMW-MAA) : A Comparative Study Using the Cyclic Anhydride of DTPA (CA) vs. the Mixed Anhydride of DTPA (MA) for Antibody Chelation." *J. Nucl. Med.* 24: P125, 1983. (abst).

270. Wang, T.S.T., Srivastava, S.C., Fawwaz, R.A., *et al.*, "A Comparison of the Cyclic Anhydride and Mixed Anhydride Methods for In-111-DTPA Chelation to Monoclonal Antibodies." *Nuklearmedizin* 4: 193, 1984.

271. Najafi, A., Childs, R.L., and Hnatowich, D.J. "Coupling Antibody with DTPA - An Alternative to the Cyclic Anahydride." *Int. J. Appl. Radiat. Isot.* 35: 554, 1984.

272. Najafi, A. and Hutchison, N. "The Use of DTPA Diactivated Ester in Coupling DTPA to Proteins." *Appl. Radiat. Isot.* 37: 548, 1986.

273. Buckley, R.G., Barnett, P., Searle, F., *et al.*, "A Comparative Distribution Study of In-111-Labelled DTPA and TTHA Monoclonal Antibodiy Conjugates in a Choriocarcinoma Xenograft Model." *Eur. J. Nucl. Med.* 12: 394, 1986.

274. Mathias, C.J., Sun, Y., Welch, M.J., *et al.*, "Targeting Radiopharmaceuticals : Comparative Biodistribution Studies of Gallium and Indium Complexes of Multidentate Ligands." *Nucl. Med. Biol.* 15: 69, 1988.

275. Som, P., Oster, Z.H., Zamora, P.O., *et al.*, "Radioommunoimaging of Experimental Thrombi in Dogs Using Technetium-99m Labeled Monoclonal Antibody Fragments Reactive with Human Platelets." *J. Nucl. Med.* 27: 1315, 1986.

276. Paik, C.H., Phan, L.N.B., Hong, J.J., *et al.*, "The Labeling of High Affinity Sites of Antibodies with Tc-99m." *Int. J. Nucl. Med. Biol.* 12: 3, 1985.

277. Paik, H.C., Ebbert, M.A., Murphy, P.R., *et al.*, "Factors Influencing DTPA Conjugation with Antibodies by Cyclic DTPA Anhydride." *J. Nucl. Med.* 24: 1158, 1983.

278. Matzku, S., Kirchgessner, H., Dippold, W.G., *et al.*, "Immunoreactivity of Monoclonal Anti-Melanoma Antibodies in Relation to the Amount of Radioactive Iodine Substituted to the Antibody Molecule." *Eur. J. Nucl. Med.* 11: 260, 1985.

279. Fawwaz, R.A., Wang, T.S.T., Estabrook, A., *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.

280. Gobuty, A.H., Kim, E.E. Weiner, R.E., *et al.*, "Radiolabeled Monoclonal Antibodies: Radiochemical Pharmacokinetic and Clinical Challenges." *J. Nucl. Med.* 26: 546, 1985.

281. Brady, L.W., Woo, D.V., Heindel, N.D., *et al.*, "Therapeutic and Diagnostic Uses of Modified Monoclonal Antibodies." *Int. J. Radiation Oncology Biol. Phys.* 13: 1535, 1987.

282. Sillen, L. and Martell, A. *Constants of Metal Ion Complexes.* Supp 1. Spec Publ No.25, London, The Chemical Society, 1971.

283. Sillen, L. and Martell, A. *Stability Constants of Metal-Ion Complexes.* Spec. Publ. No.17, London, The Chemical Society, 1964.

284. Hnatowich, D.J., Griffin, T.W., Kosciuczyk, C., *et al.*, "Pharmacokinetics of an Indium-111-Labeled Monoclonal Antibody in Cancer Patients." *J. Nucl. Med.* 26: 849, 1985.

285. Cole, W.C., Denardo, S.J., Meares, C.F., *et al.*, "Comparative Serum Stability of Radiochelates for Antibody Radiopharmaceuticals." *J. Nucl. Med.* 28: 83, 1987.

286. Ward, M.C., Roberts, K.R., Westwood, J.H., *et al.*, "The Effect of Chelating Agents on the Distribution of Monoclonal Antibody in Mice." *J. Nucl. Med.* 27: 1746, 1986.

287. Goodwin, D.A., Goode, R., Brown, L., *et al.*, "In-111 Labeled Transferrin for the Detection of Tumors." *Radiology* 100: 175, 1971.

288. Lilien, D.L., Berger, H.G., Anderson, D.P., *et al.*, "In-111 Chloride: A New Agent for bone Marrow Imaging." *J. Nucl. Med.* 14: 184, 1973.

289. Roos, J.C., Koomans, H.A. Boer, P., *et al.*, "Determination of Glomerular Filtration Rate by In-111-DTPA." *Eur. J. Nucl. Med.* 6: 551, 1981.

290. Hnatowich, D.J. "Label Stability in serum of Four radionuclides on DTPA-Coupled Antibodies - An Evaluation." *Nucl. Med. Biol.* 13: 353, 1986.

291. Nonnemacher, D. and Brossmer, R. "Effects of Chemical Modification on the Conformation and Biological Activity of . eanut Agglutinin." *Biochem. Biophys. Acta.* 668: 149, 1981.

292. Pressman, D., Day, E.D. and Blau, M. "The Use of Paired Labelling in the Determination of Tomour Localizing Antibodies." *Cancer Res.* 17: 845, 1957.

293. Thedrez, Ph., Blottiere, H., Chatal, J.F., *et al.*, "Comparison between I-131 and In-111 as Radiolabels For Monoclonal Antibodies in Immunoscintigraphy of Tumor bearing Nude Mice." *Tumor Biology* 7: 137, 1986.

294. Pimm, M.V., Perkins, A.C. and Baldwin, R.W. "Differences in Tumor and Normal Tissue Concentration of Iodine and Indium-Labelled Monoclonal Antibody." Eur. J. Nucl. Med. 11: 300, 1985.

295. Sakahara, H., Endo, K., Nakashima, T., *et al.*, "Localization of Human Osteogenic Sarcoma Xenografts in Nude Mice by a Monoclonal Antibody Labeled with Radioiodine and Indium-111." *J. Nucl. Med.* 28: 342, 1987.

296. Murray, J.L., Rosenblum, M.G., Lamki, L., *et al.*, "Clinical Parameters Related to Optimal Tumor Localization of Indium-111-Labeled Mouse Antimelanoma Monoclonal Antibody ZME-018." *J. Nucl. Med.* 28: 25, 1987.

297. Otsuka, F.L., Fleischman, J.B. and Welch, M.J. "Comparative Studies Using I-125 and In-111-Labeled Monoclonal Antibodies." *Nucl. Med. Biol.* 13: 325, 1986.

298. Otsuka, F.J. and Welch, M.J. "Evidence for a Saturable Clearance Mechanism for In-111-Labeled Monoclonal Antibodies." *Int. J. Nucl. Med. Biol.* 12: 331, 1985.

299. O'Grady, L. F., Denardo, G. and DeNardo, S. *Radiolabelled Monoclonal Antibodies for the Detection of Cancer." American Journal of Phyysiologic Imaging* 1: 44, 1986.

300. Pimm, M.V., Perkins, A.C., Armitage, N.C., *et al.*, "The Characteristics of Blood-Borne Radiolabels and the Effect of Anti-Mouse IgG Antibodies on Localization of Radiolabeled Monoclonal Antibody in Cancer Patients." *J. Nucl. Med.* 26: 1011, 1985.

301. Sands, H. and Jones, P.L. "Methods for the Study of the Metabolism of Radiolabeled Monoclonol Antibodies by Liver and Tumor." *J. Nucl. Med.* 28: 390, 1987.

302. Dassin, E., Eberlin, A., Briere, J., *et al.*, "Metabolic Fate of Indium-111 in the Rat." *Int. J. Nucl. Med. Biol.* 5: 34, 1978.

303. Mathias, C.J. and Welch, M.J. "Studies on the Entrapment of In-111 in the Liver Following Administration of Proteins Labeled Using Bifunctional Chelates." *J. Nucl. Med.* 28: 657, 1987.

304. Steinstraber, A., Schwarz, A., Kuhlmann, L., *et al.*, "Kinetics of Monoclonal Antibodies - Influence of Labeling." *J. Nucl. Med* 28: 693, 1987.

305. Rosenblum, M.G., Murray, J.L., Haynie, T.P., *et al.*, "Pharmacokinetics of

In-111-Labeled Anti-p97 Monoclonal Antibody in Patients with Metastatic Malignant Melanoma." *Cancer Research* 45: 2382, 1985.

306. Khaw, B.A., Cooney, J., Edgington, T., *et al.*, "Differences in Experimental Tumor Localization of Dual Labeled Monoclonal Antibody". *J. Nucl. Med.* 27: 1293, 1986.

307. Halpern, S.E., Stern, P.H., Hagan, P.L., *et al.*, "Stability, Characterization, and Kinetics of In-111 Labeled Monoclonal Antitumor Antibodies in Normal Animals and Nude Mouse-Human Tumor Models." *Cancer Res.* 43: 5347, 1983.

308. Shochat, D., Sharkey, R.M., Vattay, A., *et al.*, "In-111 Chelated by DTPA-Antibody is Retained in the Liver as a Small Molecular Weight Moiety." *J. Nucl. Med.* 27: 943, 1986.

309. Alvarez, V.L., Wen, M.L., Lee, C., *et al.*, "Site-Specifically Modified In-111 Labelled Antibodies Give Low Liver Backgrounds and Improved Radioimmunoscintigraphy." *Nucl. Med. Biol.* 13: 347, 1986.

310. Goldenberg, D.M. and Deland, F.H. "History and Status of Tumor Imaging with Radiolabeled Antibodies." *J. Biol. Resp. Modifiers* 1: 121, 1982.

311. Hagan, P.L., Halpern, S.E., Chen, A., *et al.*, "*In Vitro* Kinetics of Radiolabeled Monoclonal Anti-CEA Antibodies in Animal Models." *J. Nucl. Med.* 26: 1418, 1985.

312. Wilbanls, A.B., Peterson, J.A., Miller, S., *et al.*, "Localization of Mammary Tumors *In Vivo* with I-131 Labelled Fab Fragments of Antibodies Against Mouse Mammary Epithelial (MME) Antigens." *Cancer* 48: 1768, 1981.

313. Wahl, R.L., Parker, C.W. and Philpott, G.W. "Improved Radioimaging and Tumor Localization with Monoclonal $F(ab')_2$." *J. Nucl. Med.* 24: 316, 1983.

314. Goodwin, D.A., Meares, C.F. Diamanti, C., *et al.*, "Use of Specific Antibody For Rapid Clearance of Circulating Blood Background From Radiolabeled Tumor Imaging Proteins." *Eur. J. Nucl. Med.* 9: 209, 1984.

315. Epenetos, A.A. "Antibody Guided Lymphangiography in the Staging of Cervical Cancer." *Br. J. Cancer* 51: 805, 1985.

316. Goodwin, D.A., Meares, C.F., McCall, M.J., *et al.*, "Pre-Targeted Immunoscintigraphy of Murine Tumors with Indium-111-Labeled Bifunctional Haptens." *J. Nucl. Med.* 29: 226, 1988.

317. Haseman, M.K., Goodwin, D.A., Meares, C.F., *et al.*, "Metabolizable In-111 Chelate Conjugated Anti-Idiotype Monoclonal Antibody for Radioimmunodetection of Lymphoma in Mice." *Eur. J. Nucl. Med.* 12: 455, 1986.

318. Dunn, M.A., Blalock, T.L. and Cousins, R.J. "Minireview : Metallothionein." *Proc. Soc. Exp. Biol. Med.* 185: 107, 1987.

319. Wagner, M.S., Huemer, R.P., Spolter, L., *et al.*, "Radioindium Localization in Mouse Tumors." *J. Nucl. Med.* 12: 471, 1971.

320. Saha, G.B. and Boyd, C.M. "Tissue Distribution of Ga-67 Citrate and $^{111}InCl_3$ in Mouse with Adenocarcinoma." *Int. J. Nucl. Med. Biol.* 10: 223, 1983.

321. Zalutsky, M.R. and Narula, A.S. "A Method for the Radiohalogenation of Proteins Resulting in Decreased Thyroid Uptake of Radioiodine." *Appl. Radiat. Isot.* 38: 1051,

1987.

322. Lederer, M. and Shirley, V.S. (Eds.) "Table of Isotopes." 7th Ed., Wiley, New York, 1978.

APPENDIX 1

OD_{595} Versus Concentration of Bovine Gamma Globulin and PNA with the Standard Bio-Rad® Protein Assay

Protein Concentration PNA or BGG[1]	BGG[1] OD_{595}	PNA OD_{595}
0.20 mg/ml	0.12	0.09
0.50 mg/ml	0.32	0.28
0.75 mg/ml	0.45	0.41
1.00 mg/ml	0.64	0.56
1.35 mg/ml	0.80	0.75
1.60 mg/ml	0.87	0.84

[1] BGG = Bovine Gamma Globlin Standard