

AN APPROACH TO THE TECHNETIUM LABELLING
OF PROTEINS

by

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B.S., Harvey Mudd College
(1980)

SUBMITTED TO THE DEPARTMENT OF CHEMISTRY

IN PARTIAL FULFILLMENT FOR THE

DEGREE OF

DOCTOR OF PHILOSOPHY


at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

MAY, 1984

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ABSTRACT

A technique for the technetium labelling of antibodies has been developed. A range of cholesterol derivatives containing protein binding groups was synthesized and characterized. Vesicles were prepared using distearoylphosphatidyl choline and the cholesterol derivatives. The vesicles were labelled with Technetium-99m as the complex $[Tc(CNC(CH_3)_3)_6]^+$. These lipophilic cations have been well characterized and can be prepared directly from pertechnetate. The various lipophilic protein modification agents were analyzed for their protein binding ability, and two agents selected as the best. These were cholest-5-en-3 β -yl 5-carboxypentyl ether N-hydroxysuccinimide ester and Cholest-5-en-3 β -yl 6-carboxyhexyl ether N-hydroxysuccinimide ester.

The protein bound to vesicles was analyzed using a modified radioimmunoassay technique and it was found that approximately 40% of the bound antibody was viable. A cell assay involving both antigen positive and antigen negative cells along with both specific antibody and control antibody bound to vesicles showed good specificity using radioiodine labelled antibody. The technetium label did not show specificity, due to the label leaving the vesicles and binding to the cells directly.

Both sub-cutaneous and subrenal tumors were grown in mice and imaged using the vesicle-antibody complex. No specific localization in the tumor was seen. Gamma camera images showed tumor activity but the biological distribution of the labels did not confirm these observations.

Thesis Supervisor: Alan Davison
Title: Professor of Chemistry

To Rona and My Family

"Imido, but I didn't"

--TIM!

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CHAPTER I

INTRODUCTION

The research work described in this thesis has been directed towards finding a non-invasive way to detect abnormalities in a living body. The abnormalities could be tumors or their metastases, or any other tissue whose cells are altered such that they no longer behave normally. Millions of people have died due to malignant tumors and their effects.¹ Cancer research has made great strides in developing treatment programs for a wide variety of cancers,² but obviously the detection of the cancerous growth is very important in any treatment.

Exploratory surgery is a very expensive, time consuming, and hazardous procedure, which may or may not find the whole problem. A technique which could identify the number, size, and location of the tumors present without surgery would be very useful. A system that could then be adapted to a therapeutic role would be doubly useful and would enhance the treatment options currently available.

A system of non-invasive imaging is already in use for related problems. The field of nuclear medicine is a huge and growing discipline that aids in the detection of a wide variety of diseases. Dominating the field is the nuclide Tc-99m.³ This nuclide is ideally suited to diagnostic nuclear medicine applications due to its short half-life (6.02 hour) and gamma energy (140 keV).⁴ This energy is very efficiently detected by modern gamma camera equipment. The Tc-99m is easily obtained by the use of a "generator" which contains Mo-99. The Mo-99 is bound to an alumina column as molybdate, which is insoluble in saline. The 67 hour half-life beta decay produces Tc-99m as pertechnetate, which is soluble in saline. To obtain $^{99m}\text{TcO}_4^-$, one simply elutes the column with saline. The generator eluate contains both $^{99m}\text{TcO}_4^-$ and the decay product $^{99}\text{TcO}_4^-$. The isotope ^{99}Tc is also radioactive

and decays with a weak beta over a 213,000 year half-life to Ru-99. Since Mo-99 is 6% of the fission yield of Uranium, there is an ample supply of Mo-99 due to the large number of nuclear reactors in use.

The generator eluant $^{99m}\text{TcO}_4^-$ can be used as obtained or chemically modified before use. There is a large industry in supplying generators, and a larger one supplying "kits" which will alter the biodistribution of the ^{99m}Tc . Most of the kits contain a reducing agent (such as Sn(II)) and a ligand (such as pyrophosphate) so that the Tc-99m is stabilized in the lower oxidation state. The new Tc-99m complex will have a distinctly different biodistribution than $^{99m}\text{TcO}_4^-$. There are kits to make compounds that localize in bone, blood, kidneys, liver, etc. Millions of scans are performed yearly using the variety of kits for Tc-99m.

As useful as Tc-99m is, it is not the only radionuclide currently in use. The other nuclides that are used vary greatly in form and function. The aquo cation of Thallium-201 is used to image myocardial infarcts in heart attack victims. It is very useful in determining the extent of heart damage produced by the attack. Lung perfusion studies using Xenon-133 gas are a way of directly determining if areas of lungs have been damaged or collapsed. A wide range of organic molecules and proteins are labelled with Iodine-131 to follow metabolic or other functional aspects of the body. The purpose is to obtain good image quality, low dosimetry, and easy preparation. Because there are always ways of improving these variables, researchers are trying to develop new and better radiopharmaceuticals.

Other approaches to detecting internal abnormalities include Computer Assisted Tomography, more commonly known as CT or CAT scanning, and a

relatively new approach called nuclear magnetic resonance spectroscopy, commonly called NMR. CT is a method of taking X-rays of the head which give information as X-ray density in a series of slices. A source and a detector rotate around the head, collecting the data which the computer transforms into images on a screen or paper. CT is used to the extent that "CT to a neurologist is like chest X-rays to an internist".⁵

NMR is growing as a diagnostic tool and will probably grow much more as improvements and economies of scale take effect in the next few years. The technique involves placing the body in a strong magnetic field, pulsing the field with a radio-frequency field and then detecting the relaxation of the nuclei. This technique has been used extensively in the physical sciences to study small molecules, and only in the last few years been applied to medicine. The images are very good and give information of soft tissue that cannot be imaged using any other method.

With the exception of brain tumors, none of the above mentioned methods are effective at imaging tumors. Images of tumor would be very useful to the diagnosis of cancer. The problem that is encountered is finding an agent that discriminates between tumor and non-tumor tissue. Obviously there are vast differences in growth rate and morphology, but finding a specific marker is difficult.

An approach which has been pursued involves the use of antibodies. Antibodies are a component of the body's own immune system and are proteins that have a high affinity for a specific antigen. The antibodies bind to the antigen and then are removed from the body by the rest of the immune system. The antibodies are a key element since they

have the specificity to discriminate between a toxic antigen and normal or essential compounds.

Antisera against certain tissue have been prepared since before the turn of the century⁶ and applied against malignant tissue. While there is still considerable debate as to whether there are tumor specific antigens,⁷ antibodies have been used as both diagnostic and therapeutic agents for years. None of these methods were satisfactory since a high antibody titer and sufficient specificity were very hard to obtain. A better approach to making antibodies was needed.

It is known that the B cells of the spleen secrete antibodies in response to an attack on the body by an antigen.⁸ Antibodies could be obtained by inducing an animal to produce an antibody in response to an antigen. The antigen is injected periodically in increasing amounts until the animal is producing a huge amount of the required antibody.⁹

There are a number of disadvantages to this system. The antibodies produced are very dilute in the body. In spite of the fact that the spleen is producing antibodies at maximum rate, their overall concentration never gets very high (on the order of micrograms per milliliter).¹⁰ Another disadvantage is that each B cell will produce a different antibody against the antigen. The mixture of compounds will all be effective as antibodies, but they will be different, and attack different sites on the antigen. This non-homogeneity causes difficulties when one is studying the actual antibody-antigen complex and find that there are many combinations. On-going production of antibody depends on continued challenging of the animal's immune system with antigen. This causes serious toxicological problems, as the animal will at some point lose the battle against the antigen, or some

other problem, and one must start over. This makes continuing studies almost impossible, since a different animal will produce a different mix of antibodies.

A better system for the production of antibodies was developed which utilized another technology related to cell fusion. Harris and Watkins described in 1965 how a mouse cell and a human cell could be fused together using a virus to make a new cell.¹¹ This technique was developed further and it was found that polyethyleneglycol would also fuse two cells together.¹² The two techniques, antibody production and cell fusion, were coupled together to make the monoclonal antibody production technique which is in widespread use.¹³⁻¹⁷

Monoclonal antibodies of a certain type are all identical as they come from a single cell line. An animal is challenged with antigen as in the normal antibody production technique, and after it is determined that the spleen is producing antibody, the animal is killed and the spleen cells grown in culture. A myeloma (bone marrow cancer) cell line is also grown in culture, but is deficient in an essential enzyme for growth in a special culture. The cells are mixed and fused using either the virus or the polyethylene glycol technique. After fusion, the cells are placed in a medium containing the previously lethal compound. Unfused myeloma cells will die in the medium, but the fused cells are resistant. The spleen cells grow so slowly with respect to myeloma cells that they diminish quickly in proportion. The cells are grown until only fused cells exist and there is a large number of them. Colonies are checked to determine which are producing antibody. The antibody-producing colonies are diluted so that each cell is separate and then allowed to grow. The new colonies are checked and

antibody-producing cells are again diluted and then allowed to grow. If the dilution is such that only one cell is present to start a new colony, then all the cells in the colony will be identical. This cloning technique is repeated until only antibody-producing colonies exist. Colonies resulting from different original cell fusions will be produce different antibodies, but every cell in any one colony will produce exactly the same antibody.

These cells are then injected into an animal and grown into a tumor. This tumor will produce antibody at a huge rate as it grows. The antibody concentration in the bodily fluids will be very high (on the order of milligrams per milliliter)¹⁰ and can be isolated easily. Isolation involves chromatography and electrophoresis steps, and will yield a large amount of pure, homogeneous antibody. Antibodies that are produced by the clones of a single myeloma-spleen cell fusion are called monoclonal antibodies. The cell line is also maintained in culture, so new antibody-producing animals can be grown whenever needed. New techniques in cell culture allow a reasonable production of antibody without the use of an animal. This simplifies the isolation of antibody and yields a purer compound than the animal. These techniques will produce exactly the same antibody, and therefore continuity and production are assured.

The monoclonal antibodies thus produced are very useful in a number of ways. They are used for in vitro assay techniques for the determination of components of cell membranes,¹⁵ the study of kinetics of antibody action on antigens,¹⁷ and many other applications. The high specificity and homogeneity allow for more elegant and quantitative techniques to be used. A serum fraction of polyclonal

antibodies has an indeterminable amount of specific antibody. The monoclonal antibody can be quantified very accurately. This makes the in vitro applications very useful and informative, but a much greater area of application is the in vivo localization of the antibodies.

A monoclonal antibody, with its high specificity, will only bind the antigen for which it has been developed. This characteristic means that if the monoclonal antibody is injected, it will circulate in the blood until it sees the antigen. It will then bind to the antigen to form a stable antibody-antigen complex. As this continues, a greater and greater fraction of the antibody will be localized at the antigen site. If the antigen is on a tumor, then the monoclonal antibody will collect at the tumor. This concentration of antibody will probably not be enough to affect the growth of the tumor directly, but would give a lot of information to an observer if it could be followed from outside the body. The antibody could also be used to direct therapeutic doses of either chemicals or nuclides to the tumor site.

Many approaches to following the distribution of monoclonal antibodies have been developed, most of them having to do with radionuclides. The most common nuclides associated with monoclonal antibodies are the various isotopes of Iodine, (especially I-123, I-125, and I-131), In-111, Tc-99m, and others. These are all gamma emitting isotopes and have been used in nuclear medicine applications or research for many years. Iodine has a manipulative advantage in that it can be covalently bound to a protein by using well-developed techniques. There are a number of iodination techniques used to radiolabel proteins. The more common include Chloramine-T, Iodogen, Iodine monochloride, and molecular iodine.

The procedure using Chloramine-T is the most widely used iodination technique. It was developed in the early 1960's by Hunter and Greenwood,¹⁸ and is an oxidative technique. Sodium iodide is oxidized indirectly by the chloroamide compound. This produces a highly electrophilic iodine species which reacts with the nucleophilic amino acids like tyrosine and histidine.

The major drawback to Chloramine-T is the harsh oxidizing environment to which the protein is exposed. Many proteins are oxidatively unstable, and will denature under these conditions. Thiol and thioether groups are particularly sensitive to oxidation, and tryptophanyl peptide bonds will also be cleaved.¹⁹ These difficulties limit the application of Chloramine-T to more hearty proteins.

A more gentle approach which uses a similar mechanism is Iodogen.²⁰ This is also a chloroamide, but is insoluble in water. The iodogen is dissolved in an organic solvent and coated on the sides of the reaction vessel. The solvent is then evaporated to form a film. This markedly decreases the oxidizing overpotential found in the chloramine-T solution. The protein is still iodinated, but not oxidized.

Molecular iodine I_2 is used without additional reagents, and will iodinate proteins reasonably well. A major problem is converting the sodium iodide that is available to molecular iodine. A range of oxidizing agents have been used, but all lead to the same situation. Since the protein will attach to only one of the two iodines, there is an immediate 50% loss of radioactivity. Exchange of the unoxidized I^- with added non-radioactive iodine I_2 is effective, but the specific activity is diluted. The method has two major drawbacks; the

radiochemical yield is never above 50%, and molecular iodine is volatile, greatly increasing the radiation exposure to the operator.

A better approach is the use of iodine monochloride. The protein will bind to the iodine and not the chlorine, thus solving the 50% yield problem of I_2 . Usually radioiodide is added to carrier ICl and then added to the protein or organic molecule. This is a useful technique and is effective if a suitable iodination site is present. As has just been shown, there are ways of attaching the various isotopes of iodine to a monoclonal antibody. The major drawback to using iodine to follow the localization of antibody is the nuclear properties of the iodine isotopes.

The three most commonly used radioisotopes of iodine are: I-123, I-125, and I-131. Each have disadvantages that make radiopharmaceutical use less than optimal. The isotope I-131 has a relatively long half-life of 8.07 days, and gamma energies which vary from 80 to 722 keV.⁴ The useful range for radiopharmaceuticals is from 100 to 200 keV. Unfortunately, only 0.3% of the gamma intensity is in this range. Most of the activity is at 364 keV (79%) which gives a high radiation dose to the patient and a poor image. In spite of these drawbacks, I-131 has been used extensively as a label for antibodies and small organic molecules since a suitable replacement is not currently available.²¹

The isotope I-125 suffers from the opposite problem. As well as a very long 60 day half-life, the gamma energy is only 35 keV. Almost all of the gamma intensity will be absorbed by the body and essentially no image would be obtained. This isotope is not useful for radiopharmaceuticals, especially since the biological half-life is also very long.

It is very useful for in vitro labelling experiments, however, where the low gamma energy and long half-life is very useful. Since it is easy to shield, the worker gets very little dose, and the long half-life allows extended use of an iodinated compound without the need to reiodinate.

The isotope I-123 shares neither of the problems of I-125 nor I-131. It has a short half-life of 13.3 hours and has a gamma energy of 159 keV. This would seem to suggest that I-123 is ideal; but the production methods currently available always produce I-124 as a significant contaminant. The isotope I-124 has a 4.2 day half-life and a 603 keV gamma energy. This high gamma energy gives very high dosimetry and no image. If the percent contamination of I-124 could be significantly lowered, then I-123 could become a very useful radionuclide. Meanwhile, it should be avoided as an in vivo agent.

A candidate for use as a new radiopharmaceutical is In-111. This metal has a stable aquo ion in the 3+ oxidation state. This makes manipulation very easy, and In-111 can be incorporated in a number of chelates to alter its biodistribution. The 2.81 day half-life is not overly long, and 48% of the gamma intensity is centered at 172.5 keV, the other 52% is at 247 keV which is not so high that dosimetry is a problem.

The isotope In-111 has been used to label monoclonal antibodies, and reasonable images have resulted.^{22,23} The method commonly used involves diethylenetriaminepentaacetic acid anhydride (DTPA anhydride). The anhydride is a reactive site and nucleophilic groups such as amines and thiols will react to form an amide or thioester linkage. This results in a strong chelate being covalently bound to a monoclonal

antibody. The In-111 is then added to the solution and is sequestered by the DTPA. This is a promising method of analysis and will probably gain in acceptance if In-111 is ever licensed for use in humans on a routine basis. So far, the nuclide's use is purely experimental, and FDA approval may take some time.

Technetium, as was stated earlier, is already in the radiopharmacies. It has a good gamma energy and half-life, and is accepted as a viable nuclide for diagnostic imaging. The only problem to be solved is the attachment of the technetium to the antibody. There have been attempts at the technetium labelling of proteins, some with reasonable success. However, none of the methods seems to be very acceptable. The pertechnetate from the generator is reduced in the presence of the protein using either stannous ion²⁴⁻²⁶ or electrolysis.²⁷ The nature of the technetium species produced is not well characterized; since there are many possible products, a mixture probably results. Technetium has an affinity for the +4 oxidation state in aqueous solutions, forming the colloidal species $TcO_2 \cdot xH_2O$. The methods outlined above probably produce a reasonable amount of $TcO_2 \cdot xH_2O$ that simply gets trapped within the protein folding. This type of labelling is very reversible, and the technetium will simply wash out of the protein.

The technetium could bind to free thiol groups, which would be produced during reduction of the pertechnetate as well as the disulfide bonds of a protein. This would form a reasonably stable labelling, but would probably interfere with the binding capabilities of the antibody. Technetium does not have a stable aquo ion, so it is not possible to use the DTPA anhydride technique as it is used for indium.

There are possibilities in some type of ligand exchange to get the technetium into a chelate on a protein, but the details are far from worked out in the system. There does not seem to be a viable direct labelling procedure for attaching technetium to an antibody. This is unfortunate, but does not preclude technetium labelling altogether. An indirect method could be equally as effective and may have other advantages that direct labelling could never have.

The system of labelling antibodies that has been developed is an indirect method. Antibodies are bound to synthetic phospholipid vesicles, and the vesicles are labelled with a lipophilic technetium compound. Phospholipid vesicles (also known as liposomes) are spherical structures with a phospholipid bilayer as the "cell wall"; they have an aqueous core and total diameter is very variable. The liposomes used in this study are small, with an approximate diameter of 700 Å. The technetium compound could be any in the class $Tc(I)(CNR)_6$, which can be very lipophilic, depending on the R group. The chemistry of the complexes has been well worked out, and they can be prepared directly from pertechnetate at the radiopharmaceutical level. The complexes have interesting distribution properties of their own, and a first generation radiopharmaceutical kit has been developed. The complexes, depending on the R group, label normal heart tissue. This is exciting since there are many disadvantages to the present heart imaging methods. The compounds have been used experimentally in humans and good heart pictures have resulted.²⁸

The most important piece of the vesicle-antibody system is the coupling agent. This must bind a protein covalently and rapidly, as well as anchor itself in the phospholipid bilayer. Of course, some

type of spacer must be placed between the anchor and the protein binding group so the two groups do not interfere with each other. The anchor must be tightly bound and should stabilize the liposome, not destabilize it. The protein binding group needs to be reactive, but not destructive, toward the antibody. The antibody needs to retain its viability since any denaturation by the protein binding group would be harmful.

In spite of these hazards, there are a number of advantages to the system which are unique. Because every liposome can bind a large number of antibodies, there is a very good possibility that every liposome will have at least one viable antibody bound. This will direct the whole package in vivo to the antigen site. The liposome will also be able to be labelled by a large number of technetium complexes. This means that every antibody can be followed by a large number of radionuclides, and every radionuclide will be directed by at least one antibody.

This situation is unique in antibody labelling. The iodination or chelation techniques get only one or two radionuclides to an antibody, so that even if the antibody is localized, there may or may not be a signal from those nuclides. Conversely, if the antibody is denatured, the nuclides will be directed to wherever the denatured protein localizes (probably the liver to be disposed of by the body). The situation of viable direction for every nuclide and nuclides for every antibody should lead to much better images.

Of course none of this is possible without first being able to bind the antibody to the liposome. There are a number of protein binding agents available, and there are, of course, advantages and

disadvantages to each. What was needed was an agent which could be attached to an anchor for the liposome attachment, reacted relatively quickly, and did not cause much damage to the antibody.

There are five side chain groups that can be derivatized: hydroxyl groups from serine, threonine, and tyrosine; thiol groups from cysteine; carboxyl groups from aspartate and glutamate; amino groups from lysine; and guanidino groups from arginine. All of these can be modified so that another group can be attached. The various modification agents vary in their harshness of conditions necessary for reaction.²⁹

Hydroxyl groups, for example, will react with acyl chlorides in non-aqueous media, but proteins are generally not stable in solvents other than water or water-alcohol mixtures. Carboxyl groups can be esterified with an alcohol, but the acidified alcohol must be the solvent, and the reaction time is usually days. This again is very impractical. Diazo compounds with copper (II) will esterify carboxyl groups, but it is necessary to then separate the protein from the copper salts. A more useful approach involves an amine and a carbodiimide. These combine to react with the carboxyl group to form an amide bond. The only drawback is that two reagents (the amine and the carbodiimide) are needed, not just one.

If the last reaction is turned around and a carboxyl group on the modifying agent and an amine on the protein are used, a new possibility arises. The carboxylate can be converted, using a carbodiimide, to an active ester with N-hydroxysuccinimide. This will be a single reagent which will react with the protein amine groups and form an amide bond.³⁰ This will work at a pH of around 8.0 in water and is reasonably fast.

Another possibility for the binding of amino groups is a modification of the Edman end group analysis reagent.³¹ He used an aryl isothiocyanate to modify the N-terminal amine of proteins. When the protein was then hydrolyzed, the modified amino acid was known to have been at the N-terminus. The isothiocyanate reacts by forming a thiourea linkage with the amine at a slightly basic pH. An anchor could be attached to an isothiocyanate and bind to not only N-terminal amines, but also any amine in the protein.

The guanidino groups on arginines will react with 1,2-diones to form stable bis Schiff base complexes.³² Various diones which work include 1,2-cyclohexanedione, trimeric butadione,³³ and camphorquinone. The reaction half time is about 15 minutes at 40°C and pH 8-9. This method is not limited to reaction with arginines, but can also be used with lysines. The amino groups can be converted to guanidino functionalities using 3,5-Dimethyl-1-pyrazoylformamidinium nitrate.³⁴ This has been shown to not interfere with enzymatic activity of proteins, and presumably would not interfere with antibody binding activity.

The last group that can be derivatized is the thiol group of cysteine. In an antibody, the various peptide chains are held together by disulfide bonds. This means that most of the thiols are tied up in a relatively unreactive bond. There is a lot of work done with antibody fragments, however, and the thiol groups are free to complex in these. A thiol will react with alkyl halides to form a thioether, but the reaction is generally slow at moderate temperatures. Synthetically this is a useful procedure, but for protein binding it is impractical. A clearly superior thiol modifying agent is an aryl maleimide.³⁵ This

reacts very quickly (on the order of minutes) to form a thioether bond. The very reactive alkene is an excellent electrophile and the thiol simply adds across the double bond. The aryl maleimides are relatively straightforward to prepare,³⁶ and are stable in aqueous solution.

As can be seen, there are a number of ways to conjugate a protein. The better ideas include: the reaction of amines with N-hydroxy-succinimide esters to form amides, the reaction of amines with isothiocyanates to form thioureas, the reaction of guanidines with 1,2-diones to form Schiff bases, and the reaction of thiols with aryl maleimides to form thioethers. It is simply a matter of trying each of these and determining the most useful to solve the protein binding problem.

The coupling agent needs two parts however; the protein binding agent and an anchor to attach itself to the liposome. The anchor must bury itself in the phospholipid bilayer so that the protein will not simply pull it out, and it must not destabilize the liposome itself. Of course, it would be advantageous to have an anchor that actually stabilized the liposome.

The liposome stability is a critical issue. The liposomes must be stable in vivo for many hours, or perhaps days, to allow the antibody to localize in the tumor. There are a number of factors which affect stability, including lipid composition, added compounds (such as cholesterol), method of formation, temperature, and lipoproteins in sera. Each of these can seriously affect the stability of the liposomes and need to be considered carefully.

There are lipoproteins in sera, and these will destabilize the liposomes in any event. The lipid composition will change the tendency of the lipoproteins to react with the liposomes. The

temperature is also very important, but the experiments will be done in a body, which will be at 37°C for humans, and a similar value for other animals. We can affect the temperature during preinjection handling, but once in vivo the temperature is set. This leaves us with three roughly equivalently important factors which we can change: the lipid composition, the added compounds, and the method of formation.

Liposomes are made using one of two methods: mechanical agitation or ultrasonic agitation. In each case the lipid is dissolved in an organic solvent such as chloroform and placed in the reaction vessel (usually a small centrifuge tube or round bottom flask). The chloroform is evaporated under a stream of nitrogen or argon and dried in vacuo. The appropriate buffer is then added and the tube agitated.

If the mechanical agitation is used, large multilamellar vesicles are formed. Multilamellar means that there are many concentric spheres of phospholipid bilayers, as in an onion. Large is a relative term and means on the order of 1000 microns (10^{-9} meters). These are relatively stable independent of the lipid or added compounds. The reason is that the outside layer could fracture, but the vast majority of the liposome is still intact. It takes many such layer decompositions before the liposome is completely decomposed.

The major disadvantage to large multilamellar vesicles is that they are too big to easily pass through capillaries. On injection, they tend to get trapped in the lung (which has a very extensive capillary system), the liver, and the spleen (both of which remove particles from the bloodstream, including vesicles). This type of image would be useless, since the liposomes will not freely circulate and may never see the tumor, much less localize within it.

Small unilamellar vesicles are made using ultrasound. An ultrasonic probe is placed in the solution and high frequency sound waves are passed through the probe into the solution. This produces vesicles which are 50-100 microns in size and contain only one phospholipid bilayer.³⁷ For these liposomes the lipid composition and added molecules are critical to the stability. If the phospholipid bilayer is ruptured, the liposome loses all of its integrity. There is no inherent stability as in the multilamellar liposomes.

The small unilamellar vesicles will circulate freely in the bloodstream, and therefore will have a chance to see the tumor and localize within it. The liver and spleen will still recognize the liposomes as foreign and will remove them, but at a slower rate than the large liposomes.

The various lipids that are used to form liposomes have differing properties. The wide range of fatty acyl groups that can be added to the phosphatidyl choline gives the compounds a range of transition temperatures. While the melting point of different phosphatidyl cholines are essentially the same, about 230°C,³⁸ there is another transition at lower temperature. This transition is very dependent on the fatty acid groups on the molecule, and is associated with motion of the alkyl chains. It is usually close to the melting point of the fatty acid molecule. The saturated fatty acids range in melting point from 44.2°C for lauric acid (C12) to 86.0°C for lignoceric acid (C24).³⁹ The most common acids are palmitic and stearic, with melting points of 63.1 and 69.6°C, respectively. The unsaturated fatty acids have much lower melting points, in the range -49.5 to 13.4°C.

The transition temperatures are closely related, at 23°C for dimyristoylphosphatidylcholine, 37-41°C for dipalmitoylphosphatidylcholine, 50-54°C for distearoylphosphatidylcholine, and 63°C for the dipalmitoylphosphatidylethanolamine.^{40,41} The phosphatidylethanolamines generally have higher transition temperatures than the corresponding phosphatidylcholines. This transition is generally believed to be a crystal to liquid crystal transition and allows the phospholipids to move around in the bilayer. This is important for living cells to allow the membrane proteins to move about to do their respective functions. Usually animals have a mix of lipids in their cell membranes whose transition temperature is slightly above normal body temperature.

In liposomes, one does not need this mobility during handling, but it is important just after formation. Small sonicated vesicles, when formed at a temperature below their transition temperature, have structural defects. The phospholipid bilayer does not form a perfect, smooth sphere. This causes two problems; the liposomes show free exchange of ions between the inside and outside, and the liposomes will fuse together to form larger liposomes. If one heats the liposomes above the transition temperature by 10°C, in just 10 minutes the defects will be remedied.⁴² The increased phospholipid motion fills in the defects and produces a uniform, stable liposome. The vesicles can then be cooled to room temperature or 37°C and handled without fear of vesicle fusion or leakiness.

By examining the transition temperatures, one finds that distearoylphosphatidylcholine has very advantageous properties. The transition temperature is significantly above 37°C (human body

temperature), but is not so high that it increases the difficulty of handling. Incubation at 60-65°C is sufficient to completely anneal the liposomes, and at 37°C they are well into the solid phase. This characteristic leads to ease of handling and stability so that a large amount of work is done with distearoylphosphatidylcholine (DSPC).⁴³

It has been found that cholesterol derivatives which are added to the DSPC will stabilize the liposomes even further.⁴⁴ This is consistent with the fact that cell membranes also contain cholesterol and other steroidal components. The added cholesterol derivative can do many functions. It stabilizes the vesicle so that the system does not spontaneously decompose, and it can also carry a group which could direct the vesicle in vivo. The cholesterol can function as the anchor in the coupling agent which would attach proteins to the vesicle. Since the cholesterol is an integral part of the phospholipid bilayer, it is very well attached to the liposome. A side chain of the cholesterol could reach out into the surrounding solution with a protein modification agent attached and bind proteins. This gives us all the components of the coupling agent: a protein binding agent, an anchor that actually stabilizes the liposome and a connecting chain which will keep the two ends from interfering with each other.

This gives us the ability to target the liposomes. John Baideschwieler has done similar work in which the side chain has an aminomannose group attached.^{44,45} This gave the liposomes a high affinity for leukocytes in vivo.⁴⁶ Others have coupled antibody fragments to liposomes and increased the cell specificity 200-fold.⁴⁷ The concept of modifying the surface of a vesicle to direct its in vivo

distribution is thus well established. The only factor to be overcome is the actual radiolabelling of the liposomes. Others have used In-111 as the label.⁴⁵ This procedure is quite elegant in that the indium is transported across the phospholipid bilayer by an ionophore, and bound by nitrotriacetic acid once inside. This method is effective at getting a large amount of radioactivity into the liposomes, and has lead to reasonable tumor images. However, In-111 is not the radionuclide of choice for diagnostic use.

As stated earlier, technetium-99m is a very advantageous nuclide, and the derivatives of the class $Tc(CNR)_6^+$ are very lipophilic. Simply incubating the liposomes with this technetium complex will label the liposomes. This simple procedure is ideally suited for radiopharmaceutical use, since the fewer steps in a preparation, the easier to assure sterility and accuracy. A large amount of the technetium can be loaded onto the liposomes using this procedure, and good gamma camera statistics can be obtained. This is the final step in the development of a good procedure for the technetium labelling of antibodies.

The whole system is as follows. Phospholipid vesicles are prepared using sonication to form small unilamellar vesicles. The lipid layer is a mixture of DSPC and a cholesterol derivative that contains a protein modification agent on a side chain. Monoclonal antibody which is specific to a tumor antigen is bound to the liposome using the protein modification agent. The liposome is then labelled with $Tc(CNR)_6^+$ and injected into a tumor-bearing animal. The antibody directs the liposome in vivo, and the technetium-99m is the label which allows external imaging of the liposome distribution. The necessary

controls at each stage of the procedure to show the viability of the vesicles and the vesicle-bound antibody had to be developed.

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CHAPTER II

THE SYNTHESIS AND CHARACTERIZATION OF A RANGE OF
LIPOPHILIC PROTEIN MODIFICATION AGENTS

INTRODUCTION

There has been considerable interest in modifying liposomes by conjugation with sugars, proteins, etc. in an effort to utilize such liposomes for a variety of purposes. Much of this work has been directed towards increasing the stability of liposomes in vivo,¹⁻⁴ specific site direction of such liposomes,⁵⁻⁹ and their use as drug delivery systems.¹⁰⁻¹¹

Compounds have been prepared in which modified sugars are attached to the cholesterol oxygen through a six-carbon side chain.¹⁵ These thioglycosyl groups direct the liposomes formed with the cholesterol derivative in vivo. The sugars alter the surface characteristics so that leukocytes recognize the vesicles very readily.⁸ Cholesterol derivatives are also useful because they have been shown to stabilize liposomes in vivo.⁹

This study details the synthesis of a range of possible lipophilic protein modification agents using this cholesterol derivative chemistry. They will be used to bind monoclonal antibodies to liposomes. The eventual goal is to radiolabel the liposomes for nuclear medicine use.

EXPERIMENTAL

Proton NMR measurements were made on a Hitachi Perkin Elmer R-24B or Bruker 250 or 270 MHz spectrometer with CDCl_3 as solvent and tetramethylsilane as the internal calibrant. Melting points were taken on a Mel-Temp apparatus and are uncorrected. Optical rotations were measured on a Rudolph Research Autopol III automatic polarimeter. Elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, Georgia.

Prior to use, distilled water was passed through a Barnstead ultrapure D8902 cartridge, followed by redistillation in a Corning AG-1 water still. Cholesterol was obtained from the J.T. Baker Chemical Co., Phillipsburg, New Jersey. All other chemicals were reagent grade and used without further purification unless otherwise specified.

Cholest-5-en-3 β -yl 5-carboxypentyl ether(IV)

Cholest-5-en-3 β -yl 6-hydroxyhexyl ether¹⁵ (5.0 g, 10.3 mmol) was dissolved in acetone (250 ml). Jones Reagent (prepared by literature methods¹⁸) was slowly dripped into the stirred alcohol solution until the yellow color persists (4.9 ml, 96% of theoretical). The small excess of Jones Reagent was quenched with isopropanol (2 ml). The solid was filtered off and the solution was concentrated under reduced pressure. The rest of the green chromium salts were filtered off and the solvent removed. The white solid was passed down a silica gel 60H column (eluant 5% MeOH/ CH_2Cl_2) and the product recrystallized from hexane. Yield 3.1 g (60%). MP: 109-111°C. ^1H NMR (CDCl_3) δ 2.1-3.6 (m, 5H, H3, H1 and H6 of hexyl chain), 5.3 (m, 1H, H6), 10.7 (broad s, 1H, acid H). $[\alpha]_D^{25}$: $-27.8 \pm 0.2^\circ$ (c 1.00 CHCl_3).

Anal. Calcd. for $C_{33}H_{56}O_3$: C, 79.15; H, 11.27. Found: C, 79.15; H, 11.48.

Cholest-5-en-3 β -yl 6-(p-tolylsulfonyloxy)hexyl ether(VI)

Cholest-5-en-3 β -yl 6-hydroxyhexyl ether¹⁵ (22 g, 45.2 mmol) and p-toluenesulfonyl chloride (25 g, 131 mmol) were dissolved in pyridine (100 ml). The solution was stirred (4 h, 25°C) under Argon.

The reaction mixture was poured into ice water (300 ml), forming a milky suspension. The suspension was carefully extracted with small portions of CH_2Cl_2 (6 x 50 ml). The combined CH_2Cl_2 fractions are washed with water (2 x 100 ml) and dried over Na_2SO_4 . The CH_2Cl_2 was removed to leave a gum, which was crystallized from hot ethanol (100 ml) on standing (-5°C, 18 h). Crude yield 25 g (85%). The product was recrystallized from hexane. MP 63.0-64.0°C; $[\alpha]_D^{25}$ $-21.2 \pm 0.2^\circ$ (c 1.00 $CHCl_3$); NMR ($CDCl_3$): δ 2.41 (s, 3H, $PhCH_3$), 2.8-3.4 (m, 3H, H3 and H1 of hexyl chain), 3.97 (t, 2H, H6 of hexyl chain), 5.3 (m, 1H, H6), 7.25 and 7.72 (d of d, 4H, aromatic H).

Anal. Calcd. for $C_{40}H_{64}O_4S$: C, 74.95; H, 10.06; S, 5.00. Found: C, 74.91; H, 10.32; S, 4.96.

Cholest-5-en-3 β -yl 6-nitriloxyhexyl ether(VII)

Cholest-5-en-3 β -yl 6-(p-tolylsulfonyloxy)hexyl ether (3.0 g, 4.7 mmol) was dissolved in dimethylsulfoxide (125 ml). Sodium cyanide (2.26 g, 47 mmol) and sodium iodide (0.06 g, 0.47 mmol) were added, and the solution stirred at 50°C for 17 h. The solution was cooled, ether (200 ml) and water (200 ml) were added. The aqueous layer was separated and extracted with ether (200 ml). The combined ether

extracts were washed with water (200 ml) and dried over Na_2SO_4 . The ether was removed and the product was crystallized from hexane, yielding 2.09 g (90.0%) of pure product. MP: 89-90.5°C. ^1H NMR (CDCl_3) δ 2.8-3.6 (m, 5H, H1 and H6 of hexyl chain, H3), 5.3 (m, H, H6). $[\alpha]_D^{25}$: $-28.3 \pm 0.2^\circ$ (c 1.00 CHCl_3).

Anal. Calcd. for $\text{C}_{34}\text{H}_{57}\text{ON}$: C, 82.36; H, 11.59; N, 2.82. Found: C, 82.54; H, 11.69; N, 2.73.

Cholest-5-en-3 β -yl 6-carboxyhexyl ether(VIII)

Cholest-5-en-3 β -yl 6-nitriloxyhexyl ether (1.927 g, 3.89 mmol) was added to potassium hydroxide in ethanol (20% w/v, 50 ml). Water (3 ml) was added and the suspension refluxed under Argon.¹⁹ After 120 h, the solution was cooled and slowly added to aqueous HCl (4 M, 75 ml). The resulting white suspension was extracted with ether (2 x 50 ml). The combined ether fractions were washed with water (50 ml) and dried over Na_2SO_4 .

The solvent was removed to leave a white solid. Chromatography on silica gel 60H (eluant 1% MeOH in CH_2Cl_2) yielded a pure product (0.80 g, 40%). MP: 90-91°C. ^1H NMR (CDCl_3) δ 3.4 (t, 2H, H1 of hexyl chain), 5.3 (m, 1H, H6), 9.7 (broad, 1H, acid proton). $[\alpha]_D^{25}$: -28.2 ± 0.2 (c 1.00, CHCl_3).

Anal. Calcd. for $\text{C}_{34}\text{H}_{58}\text{O}_3$: C, 79.32; H, 11.36. Found: C, 79.37; H, 11.57.

Cholest-5-en-3 β -yl 6-mercaptohexyl ether(XI)

Cholest-5-en-3 β -yl 6-iodohexyl ether¹⁵ (5.0 g, 8.4 mmol) and thiourea (0.76 g, 10.0 mmol) are added to ethanol (250 ml) and refluxed for 8 h.²⁰ 1 M NaOH in 50% v/v, ethanol/water (25 ml) was added and

the cloudy solution was refluxed for 14 h. The solution was cooled, 1 M HCl (aq) (25 ml) added, and concentrated to 100 ml. Water (100 ml) and ether (100 ml) were added and the ether layer collected. The water layer was extracted with ether (100 ml). The combined ether fractions were washed with water (100 ml) and dried over Na₂SO₄. The solvent was removed and the product purified by column chromatography (silica gel 60H, 40 g, eluant CH₂Cl₂). Yield: 3.012 g (71.5%). MP: 89-91°C. ¹H NMR (CDCl₃): δ 2.8-3.6 (overlapping t and m, 4H, H1 of hexyl chain, H3, thiol H), 5.3 (m, 1H, H6). [α]_D²⁵: -27.8 ± 0.2° (c 1.00, CHCl₃).

Anal. Calcd. for C₃₃H₅₈OS: C, 78.82; H, 11.63; S, 6.38. Found: C, 78.66; H, 11.65; S, 6.27.

Cholest-5-en-3β-yl 6-(p-carboxybenzylmercapto)hexyl ether(XII)

Cholest-5-en-3β-yl 6-mercaptohexyl ether (500 mg, 0.99 mmol) was dissolved in benzene (50 ml) and α-Bromo-p-toluylic acid (1.00 g, 4.6 mmol) was partially dissolved in benzene (200 ml) and 0.086 M NaOMe in methanol (65.6 ml, 5.6 mmol) was added as the other solutions were mixed. The cloudy solution was stirred at room temperature for 45 min.

The solution was poured into water (250 ml) containing HCl (10 mmol). The benzene layer was separated, washed with water (250 ml), and dried over Na₂SO₄. The benzene was removed and the compound was passed down a silica gel 60H column (20 g, eluant 1% MeOH/CH₂Cl₂). The pure fractions were isolated, and the impure fractions passed down another silica gel 60H column (10 g, eluant 1% MeOH/CH₂Cl₂). The pure fractions from both columns were combined to yield 0.327 g of product (51.6%). MP: 146-148°C. ¹H NMR (CDCl₃): δ 2.9-3.3 (m, 1H, H3), 3.4

(t, 2H, H1 of hexyl chain), 3.7 (s, 2H, S-CH₂-phen), 5.3 (m, 1H, H6), 7.5 and 8.1 (d of d, 4H, phenyl H). $[\alpha]_D^{25}$: $-21.6 \pm 0.2^\circ$ (c 1.00, CHCl₃).

Anal. Calcd. for C₄₁H₆₄O₃S: C, 77.31; H, 10.13; S, 5.03. Found: C, 77.25; H, 10.15; S, 5.00.

Cholest-5-en-3 β -yl 6-(p-carboxybenzylmercapto)hexyl ether,
N-hydroxysuccinimide ester(XIII)

Cholest-5-en-3 β -yl 6-(p-carboxybenzylmercapto)hexyl ether (150 mg, 0.24 mmol) and N-hydroxysuccinimide (30 mg, 0.26 mmol) were dissolved in CH₂Cl₂ (25 ml) and cooled to 0°C. Dicyclohexylcarbodiimide (60 mg, 0.28 mmol) was dissolved in CH₂Cl₂ (20 ml) and added to the cooled solution. The resulting solution was allowed to stand at 0°C for 2 h. The precipitate was filtered off and the solvent removed from the filtrate. The product was passed down a silica gel 60H column (3 g, eluant 1% MeOH/CH₂Cl₂). The yield of pure product was 0.167 g (96.6%). MP: 102-105°C. 270 MHz ¹H NMR (CDCl₃): δ 2.37 (t, 2H, H6 of hexyl chain), 2.91 (s, 4H, succinimide), 3.12 (m, 1H, H3), 3.39 (t, 2H, H1 of hexyl chain), 3.74 (s, 2H, S-CH₂-phen), 5.34 (m, 1H, H6), 8.08, 7.46 (d of d, 4H, phenyl H). $[\alpha]_D^{25}$: $-22.0 \pm 0.2^\circ$ (c 1.00, CHCl₃).

Anal. Calcd. for C₄₅H₆₇O₅NS: C, 73.63; H, 9.20; N, 1.91; S, 4.37. Found: C, 73.40; H, 9.30; N, 1.94; S, 4.37.

Cholest-5-en-3 β -yl 5-carboxypentyl ether, N-hydroxysuccinimide ester(V)

This was prepared similarly to cholest-5-en-3 β -yl 6-(p-carboxybenzylmercapto)hexyl ether, N-hydroxysuccinimide ester (vide supra). MP: 97-99°C. 270 MHz ^1H NMR (CDCl_3) δ 2.62 (t, 2H, H6 of hexyl chain), 2.84 (s, 4H, succinimide H), 3.13 (m, 1H, H3), 3.47 (t, 2H, H1 of hexyl chain), 5.34 (m, 1H, H6). $[\alpha]_{\text{D}}^{25}$: $-24.2 \pm 0.2^\circ$ (c 1.00, CHCl_3).

Anal. Calcd. for $\text{C}_{37}\text{H}_{59}\text{O}_5\text{N}$: C, 74.33; H, 9.95; N, 2.34. Found: C, 74.60; H, 10.06; N, 2.26.

Cholest-5-en-3 β -yl 6-carboxyhexyl ether, N-hydroxysuccinimide ester(IX)

This was prepared similarly to cholest-5-en-3 β -yl 6-(p-carboxybenzylmercapto)hexyl ether, N-hydroxysuccinimide ester. MP: 116-119°C. 250 MHz ^1H NMR (CDCl_3): δ 5.34 (m, 1H, H6), 3.45 (t, 2H, H1 of hexyl chain), 3.12 (m, 1H, H3), 2.84 (s, 4H, succinimide H), 2.61 (t, 2H, H6 of hexyl chain). $[\alpha]_{\text{D}}^{25}$: $-22.7 \pm 0.2^\circ$ (c 0.80, CHCl_3).

Anal. Calcd. for $\text{C}_{38}\text{H}_{61}\text{O}_5\text{N}$: C, 74.59; H, 10.05; N, 2.29. Found: C, 74.77; H, 10.18; N, 2.27.

Cholest-5-en-3 β -yl 6-phthalimidoheptyl ether(XIV)

The method of Landini and Rolla¹⁶ for the preparation of alkyl phthalimides was used in this preparation.

Cholest-5-en-3 β -yl 6(p-tolylsulfonyloxy)hexyl ether (30 g, 46.8 mmol) was dissolved in toluene (100 ml). Potassium phthalimide (10.8 g, 58.3 mmol) was added but did not dissolve. Hexadecyl-tri-n-butyl-

phosphonium bromide (3.0 g, 5.9 mmol) was added as a phase transfer catalyst. The suspension was stirred and heated to 95°C for 4 h. The suspension was cooled, filtered and the precipitate washed with ether (50 ml). The resulting solution was passed down a silica gel column (gel 60H, 50 g, eluant ether). The eluant was washed with 10% sodium hydroxide (100 ml), water (100 ml) and dried over sodium sulfate. The ether was removed to leave a gum. The gum was crystallized from ethanol. Yield 21.5 g (74.6%). MP: 76-78°C, NMR (CDCl₃): δ 2.8-3.3 (m, 1H, H₃), 3.4 and 3.55 (overlapping t, 4H, H₁ and H₆ of hexyl chain), 5.3 (m, 1H, H₆), 7.67 (m, 4H, aromatic H). $[\alpha]_D^{25}$: -22.7 \pm 0.2° (c 1.00 CHCl₃).

Anal. Calcd. for C₄₁H₆₁O₃N: C, 79.95; H, 9.98; N, 2.27. Found: C, 79.85; H, 10.10; N, 2.17.

Cholest-5-en-3 β -yl 6-aminohexyl ether hydrochloride(XV)

Cholest-5-en-3 β -yl 6-phthalimidoethyl ether (21.5 g, 34.9 mmol) was dissolved in hot ethanol (200 ml). The solution was heated to reflux and hydrazine hydrate (2.2 ml, 44.9 mmol) was added. The solution was refluxed under Argon for 4 h.

Dichloromethane (200 ml) and sodium hydroxide (1.0 M (aqueous), 150 ml) were added to the cooled suspension. The dichloromethane layer was collected and the aqueous phase was extracted with dichloromethane (100 ml). The combined dichloromethane layers were washed with water (2 x 100 ml) and dried over sodium sulfate. The solvent was removed under reduced pressure, ether added to dissolve the gum, and an excess of HCl (g) was bubbled through the solution. The solvent was removed and the resulting gum was crystallized from ether. Yield: 14.4 g

(78.8%). MP: 165° (dec). $[\alpha]_D^{25}$: $-24.4 \pm 0.2^\circ$ (c 1.00, CHCl₃). NMR (CDCl₃): 2.8-3.6 (m, 5H, H3, H1 and H6 of hexyl chain), 5.3 (m, 1H, H6), 7.5 (m, 3H, RNH₃⁺).

Anal. Calcd. for C₃₃H₆₀ONCl: C, 75.89; H, 11.58; N, 2.68; Cl, 6.79. Found: C, 75.90; H, 11.60; N, 2.66; Cl, 6.83.

Cholest-5-en-3 β -yl 6-(isothiocyanato)hexyl ether(XVI)

A modification of the Staab and Walther¹⁷ synthesis for alkyl isothiocyanates was used in this preparation. Cholest-5-en-3 β -yl 6-aminoethyl ether hydrochloride (4.0 g, 7.6 mmol) was added to ether (25 ml). Thiocarbonyldiimidazole (1.36 g, 7.6 mmol) and diisopropylethylamine (0.98 g, 7.6 mmol) were added. The reaction was stirred (18 h, 25°C). The solution was washed with water and dried over sodium sulfate. The solution was evaporated to an orange oil (25 mm Hg, 40°C). Repeated silica gel (60H) column chromatography yielded a colorless oil. A small amount of the oil was taken up in acetone (3 ml) and water (3 ml) added to form an oil. Scratching with a glass rod crystallized the product. The resulting crystals were used as seed crystals for the entire reaction. The mixture precipitated on standing at 5°C. Yield: 2.1 g (50%). MP: 40-42°C. 270 MHz ¹H NMR (CDCl₃) showed the expected pattern. δ 5.30 (broad, 1H, H6), 3.44 (d of t, 4H, H1 + H6 of hexyl chain), 3.07 (m, 1H, H3). $[\alpha]_D^{25}$: $-26.4 \pm 0.2^\circ$ (c 1.00 CHCl₃).

Anal. Calcd. for C₃₄H₅₇ONS: C, 77.36; H, 10.88; N, 2.65; S, 6.07. Found: C, 77.28; H, 10.93; N, 2.45; S, 5.68.

Cholest-5-en-3 β -yl 6-(m-maleimidobenzamido)hexyl ether(XVII)

Cholest-5-en-3 β -yl 6-aminoethyl ether hydrochloride (1.0 g, 1.9 mmol), m-Maleimidobenzoic succinimide¹³ (0.8 g, 2.5 mmol), and diisopropylethylamine (0.3 ml, 2.2 mmol) were dissolved in 10% CH₂Cl₂/THF (50 ml). The yellow solution was stirred at room temperature for 1 h. The solvent was removed and the resulting orange oil was passed down silica gel 60H (5 g, eluant ether). The yellow product was crystallized from acetone, crude yield 1.1 g (84%). The compound was recrystallized from acetone to yield a white product. MP: 149-152°C. 270 MHz ¹H NMR (CDCl₃) δ 3.12 (m, 1H, H3), 3.43 (overlapping t, 4H, H1 and H6 of hexyl chain), 5.34 (m, 1H, H6), 6.43 (t, 1H, amide), 6.85 (s, 2H, maleimide), 7.78-7.44 (m, 4H, phenyl H). $[\alpha]_D^{25}$: -20.6 \pm 0.2 (c 1.00, CHCl₃).

Anal. Calcd. for C₄₄H₆₄O₄N₂: C, 77.15; H, 9.42; N, 4.09. Found: C, 77.07; H, 9.72; N, 4.01.

RESULTS AND DISCUSSION

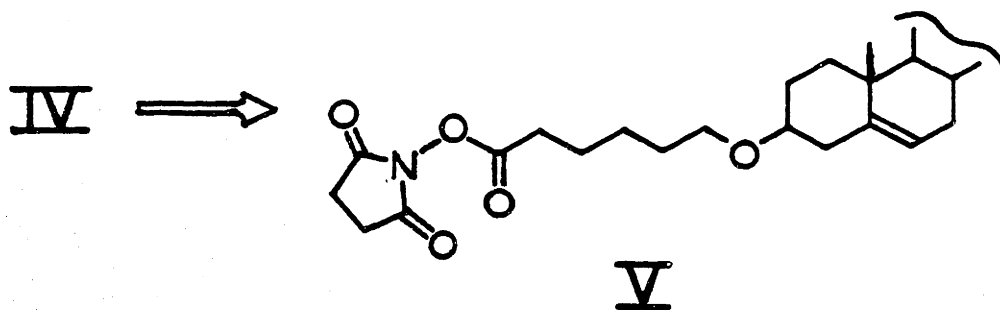
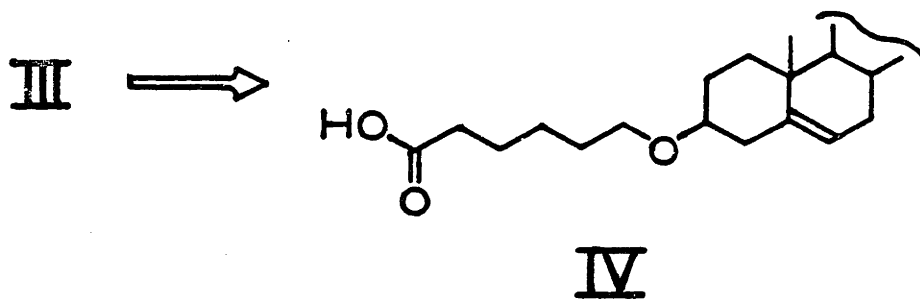
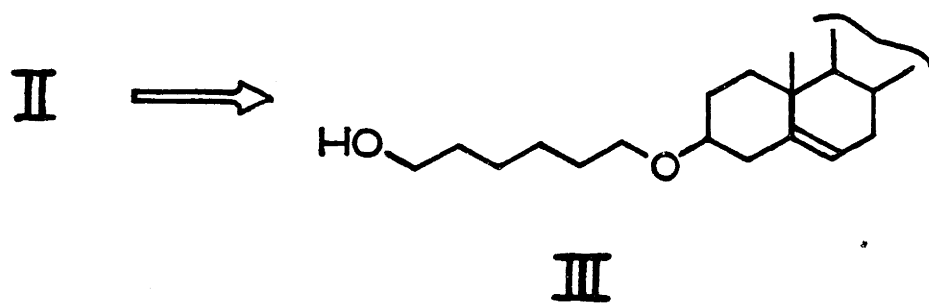
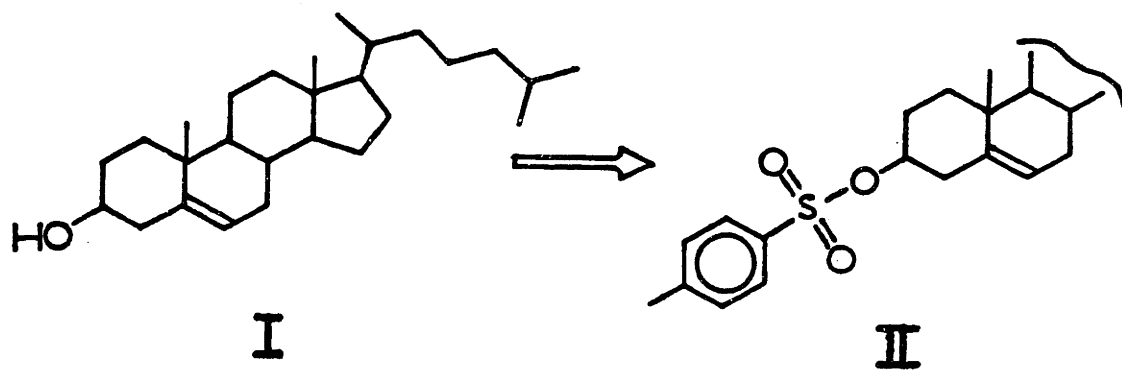
The various syntheses were performed following the Schemes 1-5. The 5-carboxypentyl cholesteryl ether succinimide (V, Scheme 2-1) was prepared starting with cholesterol (I). The cholesterol was tosylated in pyridine to yield cholesteryl tosylate (II). This was reacted with 1,6-hexanediol to form the 6-hydroxyhexyl cholesteryl ether (III). This was oxidized to the carboxylic acid (IV) using Jones' reagent. The final compound (V) was prepared as the N-hydroxysuccinimide ester using dicyclohexylcarbodiimide (dcc).

The closely related compound 6-carboxyhexyl cholesteryl ether succinimide (IX, Scheme 2-2) was prepared by a different route. The 6-tosylhexyl cholesteryl ether (VI) was prepared from 6-hydroxyhexyl cholesteryl ether (III) with p-toluenesulfonyl chloride in pyridine. The isolation of this compound was difficult and is discussed later. The tosyl compound (VI) was converted to the nitrile (VII) using sodium cyanide and a catalytic amount of sodium iodide. The nitrile was hydrolyzed completely in aqueous ethanolic base to the carboxylic acid (VIII). It was then converted to the N-hydroxysuccinimide ester (IX) using dcc as before.

The aromatic ester (XIII, Scheme 2-3) was prepared by converting the tosylate (VI) to an iodoethyl cholesteryl ether (X) using sodium iodide in acetone. The iodo complex is converted to the thiol using a two-step procedure involving thiourea as a nucleophile to form the thiuronium iodide, followed by attack by hydroxide ion to form urea, iodide, and the thiolate (XI). This reacted with methanolic sodium methoxide in

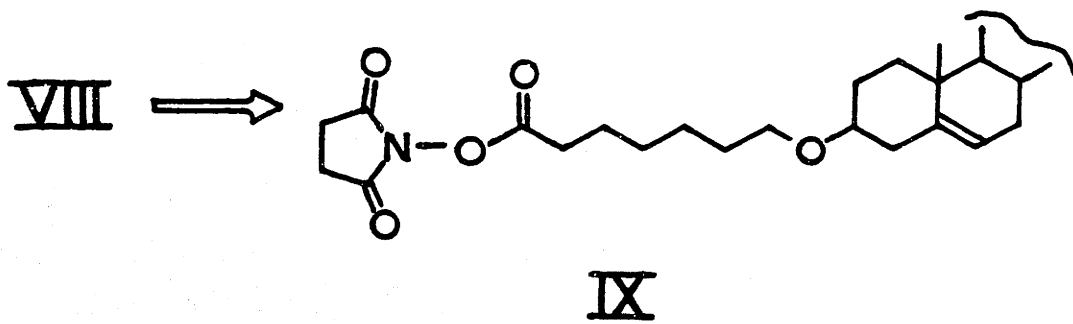
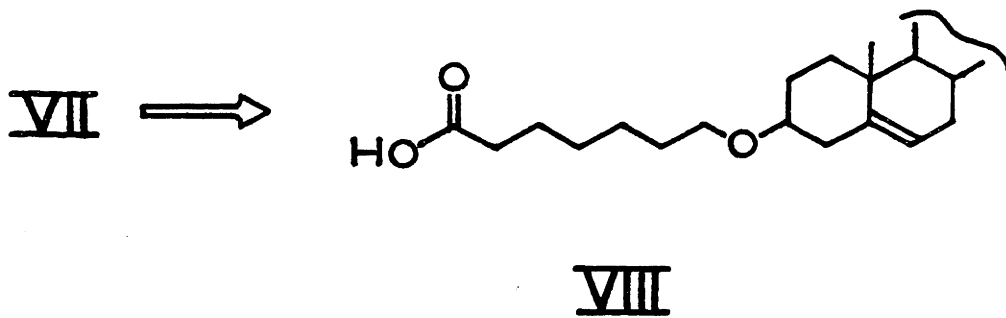
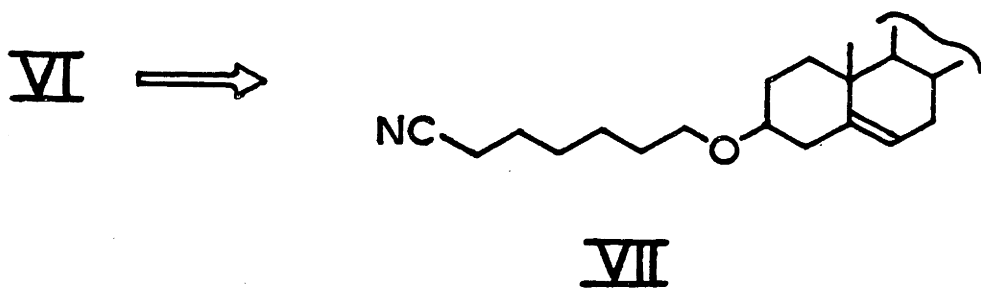
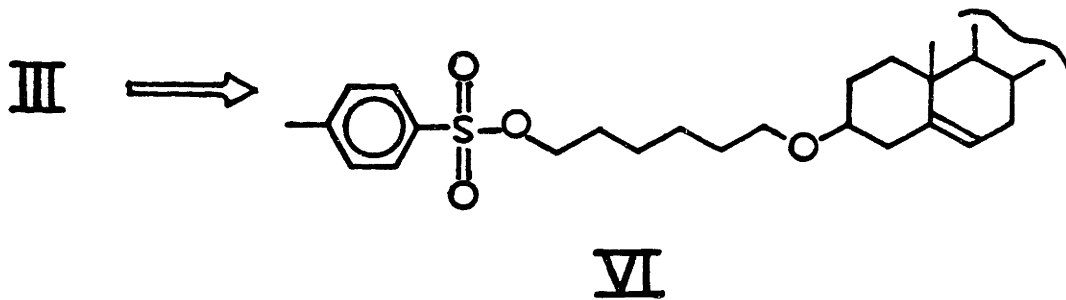
Scheme 2-1

Synthesis of Cholesteryl 5-Carboxypentyl ether, N-hydroxysuccinimide Ester.



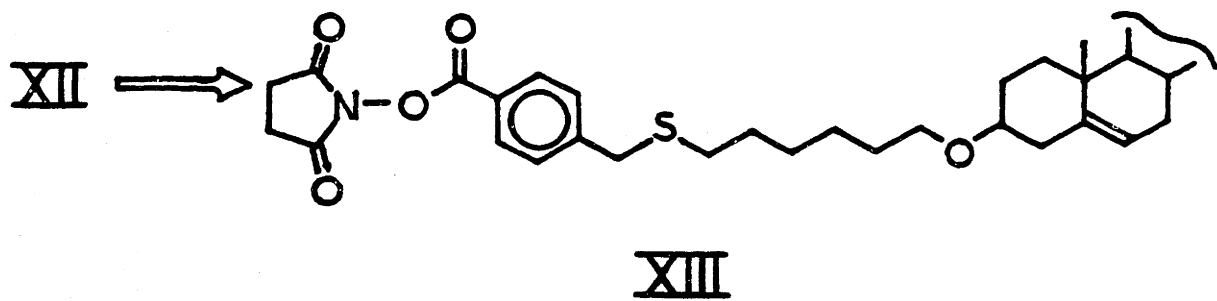
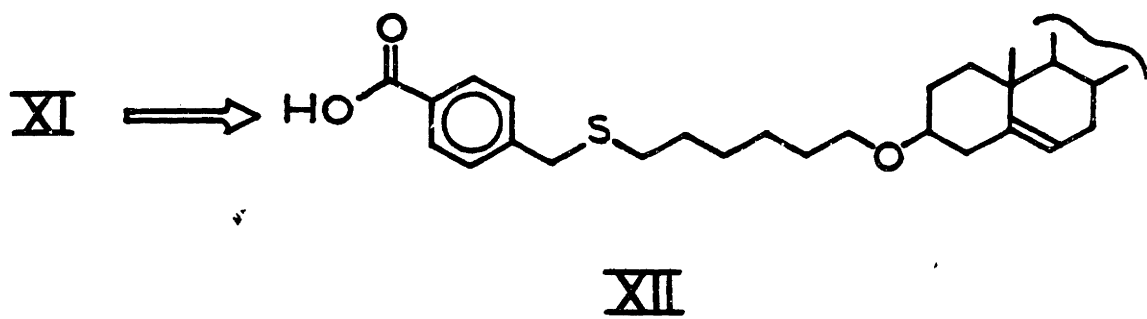
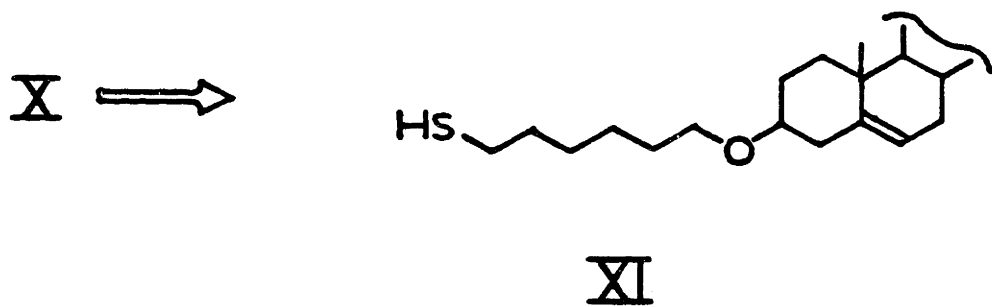
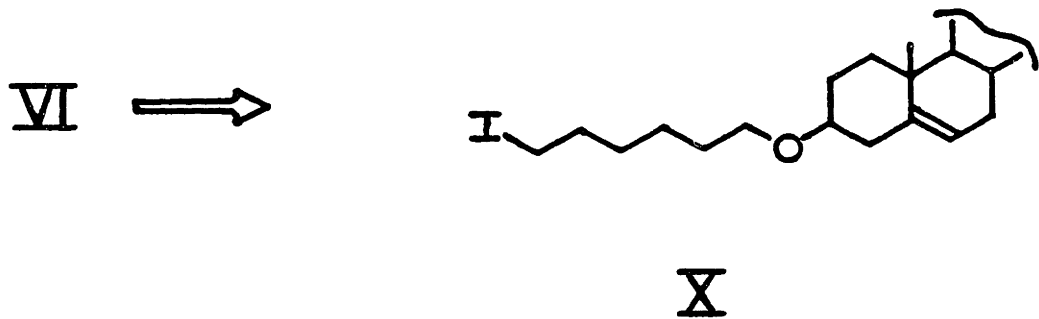
Scheme 2-2

Synthesis of Cholesteryl 6-Carboxyhexylether, N-hydroxysuccinimide Ester.



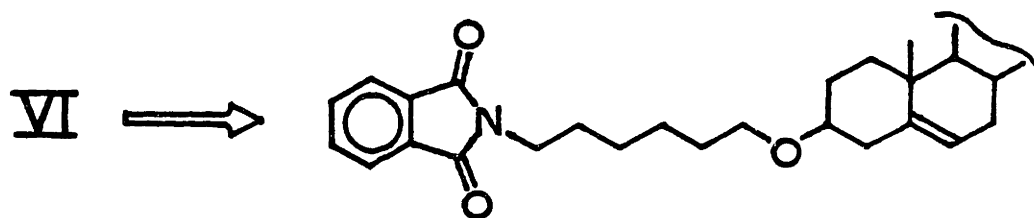
Scheme 2-3

Synthesis of Cholesteryl 6-(p-carboxybenzylmercapto)hexyl ether
N-hydroxysuccinimide Ester.

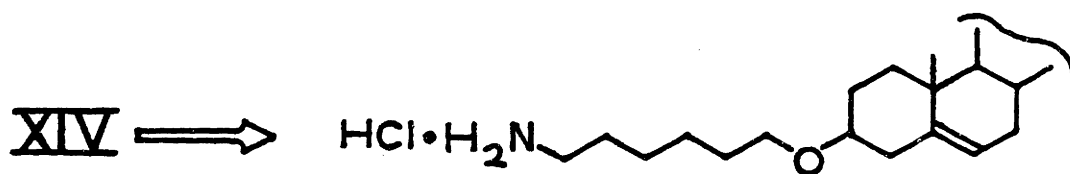


Scheme 2-4

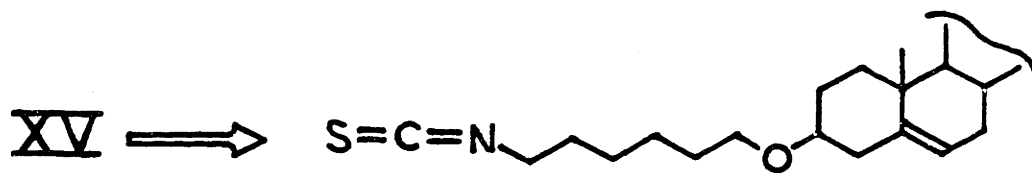
Synthesis of Cholesteryl 6-isothiocyanatohexyl Ether.



XIV



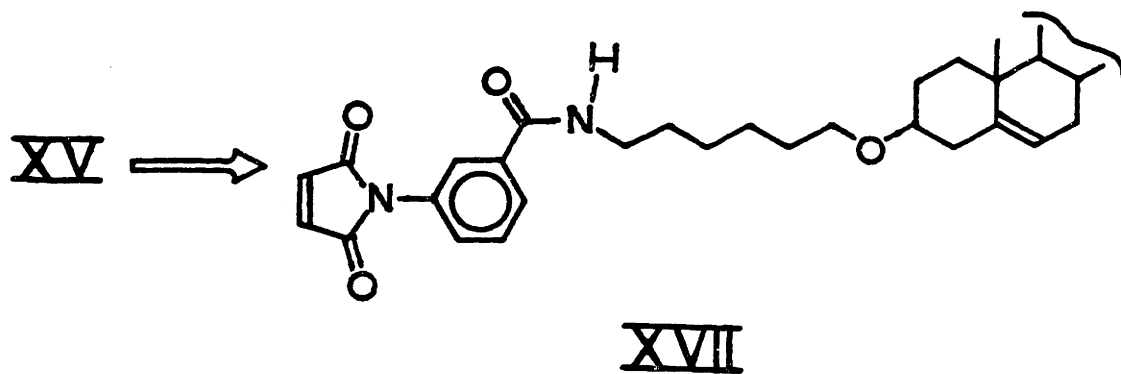
XV



XVI

Scheme 2-5

Synthesis of Cholesteryl 6-(m-maleimidobenzamido)hexyl Ether.



benzene and α -bromo-p-toluylic acid to make the aryl acid thioether compound (XII). The N-hydroxysuccinimide ester (XIII) was synthesized in the standard manner using dcc.

The isothiocyanatohexyl cholesteryl ether (XVI, Scheme 2-4), was formed from the hexyl tosylate (VI). The tosylate was first converted to the phthalimide (XIV) using potassium phthalimide and hexadecyltri-n-butylphosphonium bromide. The phosphonium salt is a phase transfer catalyst that solubilized the phthalimide in the toluene solvent. The phthalimide compound (XIV) was hydrazinolized to form the amine, which was best handled as the hydrochloride salt (XV). The amine was then reacted with thiocarbonyldiimidazole (a thiophosgene replacement) to form the isothiocyanate (XVI).

Finally, the m-maleimidobenzamidoethyl cholesteryl ether (XVII, Scheme 2-5) was formed by reacting the amine (XV) with m-maleimido-benzoic succinimide. This succinimide is a heterobifunctional protein modification agent that binds amines on one end and thiols on the other.

In the syntheses, common intermediates were used whenever possible. The final products all have an active group which has an affinity for proteins. The only difference between XIII and XVII is one methylene group. This was an artifact of the synthetic procedure, but made a rough determination of the sensitivity of protein binding to chain length possible.

Many of the compounds are liquid crystals, III and IV have large mesomorphic states, existing over a 15° temperature range. The other compounds have mesomorphic ranges from essentially zero to 10°C.

The separation during the synthesis of IV is very sensitive and great

care must be taken to ensure that emulsions do not form. Once formed they do not clear for many hours, even with traditional deemulsification techniques such as saturated brine, gentle agitation with a glass rod, or filtration. The presence of pyridine and the soapy cholesterol derivative combine to form an easily emulsified solution. Methylene chloride must be simply poured through the solution for the first few portions. Then gentle swirling should be used. Not until the last portion can the mixture be shaken safely. The water wash which follows should be handled carefully as well, gentle swirling is all that can be safely used.

The final products are all relatively low melting, which is an indication of their fatty liquid crystal properties. The characteristic proton NMR was used to determine the authenticity of the compounds prior to further analysis. Thin layer chromatography was used extensively throughout the syntheses to determine the purity and identity of the various derivatives.

The compounds were essentially insoluble in polar solvents, and this created problems synthetically. The N-hydroxysuccinimide esters are hydrolytically unstable, so they are protected from moisture. All of the compounds will slowly air oxidize, especially in the presence of ultraviolet light, so they are all stored under argon and protected from light in brown jars when applicable.

The compounds can be divided into three groups: the N-hydroxysuccinimide esters, the isothiocyanate, and the maleimide. N-hydroxysuccinimide esters have been used in peptide synthesis and protein binding.^{12,13} The active esters react quickly and cleanly with amines to form an amide bond. This is performed in aqueous solution

and mild base (pH 8). A protein usually contains a large number of free amines, giving a wide range of coupling sites. The isothiocyanate group was originally used for N-terminal protein analysis.¹⁴ Free amines react to form thiourea linkages in mild conditions (pH 8-9, 0°C). Of course, not only the N-terminal amines will react, but so will any other amine, giving the same versatility as the N-hydroxy-succinimide esters. The maleimide group reacts with thiols to form a thioether linkage. This reaction is very fast and selective.¹³ Immunoglobulines are held together by disulfide bonds, which tends to tie up the available thiol groups. However, there are some free thiols and these will react.

Each protein modification agent forms a different linkage to the protein, and has a different reactivity. The combination of NMR, IR, and mass spectra along with elemental analysis and optical rotation assures that the various compounds have been correctly synthesized and identified. The complexes are then evaluated in terms of total binding and rate of binding.

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CHAPTER III

DETERMINATION OF THE PROTEIN BINDING EFFICIENCY OF THE
CHOLESTEROL DERIVATIVES AND THE
VIABILITY OF THE BOUND ANTIBODY

INTRODUCTION

The work described in this chapter details: (i) the preparation of modified cholesteryl-phospholipid vesicles; (ii) the determination of the degree of binding of monoclonal antibodies to such vesicles; (iii) the assessment of viability of the protein-vesicle complex with respect to antigen-antibody interaction; and lastly (iv) the radiolabelling of such vesicles with a technetium-99m isonitrile complex. As an indication of the quantities and concentrations involved in using vesicle-protein conjugates as radiopharmaceutical tracers, a sample calculation is shown in Figure 3-1.

EXPERIMENTAL

The vesicles were made using a Branson Sonifier model W200P equipped with a titanium microtip. The ^{125}I activity was counted on a Packard Auto-Gamma 500 counter. Centrifugations were carried out on either a tabletop Fisher Safety Centrifuge (400 xg) or a refrigerated International Centrifuge model PR-2 (1000 xg).

Iodogen¹ was obtained from Pierce Chemical Company, Rockford, IL. Human IgG, Sepharose 4B and Sephadex G-50-80 were obtained from the Sigma Chemical Company, St. Louis, MO. Columns were preconditioned with human IgG in the appropriate buffer to block any non-specific binding sites. Distearoylphosphatidylcholine was obtained from Calbiochem, LaJolla, CA. ^{125}I as NaI was obtained from Amersham, Arlington Heights, IL. RIANEN number NEA-080 radioimmunoassay kit for the determination of prostatic acid phosphatase (PAP) and monoclonal mouse anti-prostatic acid phosphatase (maPAP) were obtained as gifts from New England Nuclear, Billerica, MA. This kit contained iodinated PAP, a polyclonal anti-PAP antibody, and a second antibody which precipitated the first antibody-antigen complex. Goat anti-mouse IgG was obtained from Cappel Laboratories, West Chester, PA.

Distilled water was passed through a Barnstead Ultrapure demineralizing cartridge and distilled using a Corning MegaPure still prior to use. The isotonic buffers (borate buffered saline (BBS) at pH 8.0 and phosphate buffered saline (PBS) at pH 6.7) were prepared fresh periodically from reagent grade chemicals. All other reagents were used as received except where noted.

All manipulations were carried out in laboratories licensed for the use of ^{125}I .

Iodination Procedure for Proteins

Iodogen¹ (1 mg) was dissolved in CH₂Cl₂ (10 ml) and 100 μl of this solution placed in a small vial. The CH₂Cl₂ was evaporated under a stream of Argon.

The Iodogen tube was placed in an ice bath and 100 μl of a 2.5 mg/ml solution of protein was added followed by 10 μl of Na ¹²⁵I and the solution mixed. The reaction was allowed to proceed for ten minutes. The solution was removed from the vial and run down a preconditioned Sephadex G-50-80 column (1 x 15 cm, eluant BBS). Fractions were collected and the first radioactive peak contained the protein. Unbound ¹²⁵I- came out later and was discarded.

The iodinated protein was stored at 2°C.

The Preparation of Unilamellar Vesicles

The cholesterol derivative (2 mg) and Distearoylphosphatidylcholine (DSPC) (16 mg) were combined and dissolved in CHCl₃ (1.50 ml). This was then distributed to 3 three milliliter Pyrex[®] centrifuge tubes (0.50 ml each). The CHCl₃ was evaporated under a stream of Argon while in a warm water bath. The tubes were dried further by storing under vacuum overnight.

An aliquot of borate buffered saline (BBS) (0.75 ml) was added to each tube. The solutions were sonicated at 100 w for 5 minutes. The tubes were cooled by immersion in glycerol during the sonication. The tubes were then incubated for 10 minutes at 60°C to "anneal" the vesicles.² The tubes were centrifuged at room temperature (400 xg for 30 minutes prior to the binding studies or 1000 xg for 30 minutes prior to the RIA procedures) The supernatant was then used for incubation.

The Binding of Proteins to Vesicles

The supernatant solution from the vesicle preparation was placed in a 15 x 85 mm culture tube. To this was added the protein solution (0.50 ml) and the tube was agitated to mix the contents. The tube was incubated at 37°C for 1 hour. The resulting mixture was subjected to size exclusion gel-chromatography on a preconditioned Sepharose 4B column (1 x 15 cm; eluant phosphate buffered saline). The vesicles appeared at the void volume of the column, the protein somewhat later. When the proteins were iodinated, the fractions were counted in a gamma counter to determine the binding efficiencies. Usually 90% of the radioactivity would elute from the column.

The Determination of the Percentage Activity of Antibody Bound to Vesicles

Three identical supernatants from vesicle preparations were separately incubated with BBS (0.50 ml) (preparation 1), iodinated mouse anti-prostatic acid phosphatase ($m\alpha$ PAP) (0.50 ml) (preparation 2), and uniodinated $m\alpha$ PAP (0.50 ml) (preparation 3). After 1 hour tube 2 was counted and then all three vesicle solutions were chromatographed on Sepharose 4B columns (1 x 15 cm, eluant PBS). The fractions from tube 2 were recounted to determine the protein binding as previously described.

The vesicle fractions from preparations 1-3 were adjusted such that the total volume in each was the same (ca. 1.5 ml).

Prior to incubation with the antigen, (iodinated prostatic acid phosphatase) the following sample tubes were prepared. Duplicate aliquots (0.2 ml) from the non-specific vesicle preparation (preparation 1) and the $m\alpha$ PAP vesicle preparation (preparation 3) were

placed in culture tubes (15 x 85 mm) and a solution of PBS (0.2 ml) was added to each.

A set of standard tubes was prepared as follows.

A sample of uniodinated $m\alpha$ PAP (2.5 mg per ml) was serially diluted in PBS solution (1:5, 1:10, 1:50, 1:100, 1:500). Duplicate samples (0.2 ml) of these standards together with a duplicate of a non-specific standard (0.2 ml PBS) were diluted with PBS (0.2 ml).

Aliquots of iodinated prostatic acid phosphatase (PAP) (0.1 ml) were added to each of the samples and standards prepared above. The contents of each tube was mixed by agitation and then allowed to stand at room temperature for 4 hours.

Four samples (0.2 ml) of the iodinated $m\alpha$ PAP vesicle solution (from preparation 2) were counted to determine the percentage of the total vesicles that were contained in a 0.2 ml sample. A PBS solution (0.3 ml) was added to each of these four tubes.

A sample of iodinated $m\alpha$ PAP was serially diluted in PBS solution in an identical fashion to that of the uniodinated $m\alpha$ PAP standards. Since $m\alpha$ PAP sticks to glass to a certain extent, the actual concentrations of the standards were corrected using the ^{125}I - $m\alpha$ PAP values. Duplicate tubes corresponding to the standards were prepared using the iodinated $m\alpha$ PAP. To each was added 0.3 ml of PBS and they were allowed to stand for 4 hours at room temperature after mixing.

Diluted goat anti-mouse IgG (0.5 ml, 1:20 dilution) was added to each of the standards and the iodinated $m\alpha$ PAP tubes described above. Aliquots of the second antibody from the RIANEN kit #NEA-080 (0.50 ml) was added to the vesicle samples obtained from preparations 1 and 3 and to 2 of the 4 iodinated $m\alpha$ PAP vesicle fractions obtained from

preparation 2. The other two tubes of iodinated m α PAP vesicles were treated with PBS (0.50 ml).

All of the tubes were allowed to stand at room temperature for ten minutes prior to centrifugation (1000 xg) at 4°C for 10 minutes. The supernatant solution was discarded and the tubes were counted.

All the standards had the non-specific counts subtracted from them to find the binding due to the m α PAP. They were then divided by the percentage precipitation determined from the iodinated m α PAP. The counts from the non-specific vesicles (preparation 1) were subtracted from the counts from the m α PAP vesicles (preparation 3) to determine the binding due to the attached m α PAP. This result was divided by the percentage precipitation determined from the iodinated m α PAP vesicles (preparation 2). The value computed was compared with the standard curve and the concentration of active antibody in 0.2 ml of the antibody preparation was determined. This value enables the concentration of viable antibody in the whole vesicle preparation to be determined.

The total antibody on the vesicles was determined by the binding study done on preparation 2 and the initial concentration of antibody. The total active antibody was divided by the total antibody to get the percent active antibody on the vesicles.

The Labelling of Vesicles with Technetium-99m

Samples of vesicles in saline were prepared in the usual way. A Zn isonitrile kit³ was allowed to thaw to room temperature. Generator eluant ^{99m}TcO₄⁻ was used to reconstitute a NEN Glucoscan™ kit and briefly shaken. A portion (0.8 ml) of the isonitrile kit was added to

the Glucosan™ vial and again briefly shaken. The vial was suspended three quarters submerged in a boiling water bath for 15 minutes. It was then allowed to cool to room temperature for 15 minutes.

The vial was counted for activity, and then the contents discarded. Water (10 ml) was added and the vial inverted twice. The water was removed and the vial counted again. The vesicle solution was added to the "empty" kit and agitated for 15 minutes. The vesicle solution was removed and both the vesicle solution and the vial were counted to enable the labelling efficiency to be determined.

RESULTS AND DISCUSSION

The determination of which protein binding agent was the most effective was undertaken. Preliminary experiments showed that new, preconditioned Sepharose 4B columns were necessary for good performance. Sepharose 4B is a relatively fragile size exclusion gel and after freezing, excess pressure, bacterial growth, or prolonged storage at room temperature will no longer have the characteristics it originally possessed. In addition to its fragile nature, it also has non-specific binding sites for protein and vesicles. A column that was freshly prepared would bind a significant fraction of the first vesicles and protein passed through it. This necessitated a preconditioning step in which vesicles and protein were passed down the column prior to its use in an experiment.

The modification agent analysis was performed using human IgG as the protein throughout. This was to eliminate variations due to protein differences. Depending on a number of factors, proteins have an affinity for vesicles without using a protein binding agent. This is probably a non-covalent interaction between lipophilic portions of the protein and the phospholipid bilayer. Different proteins have different lipophilicities, and therefore showed different non-specific binding to the vesicles. It was found that monoclonal antibodies had lower non-specific binding than the polyclonal human IgG (Table 3-1). This binding was due to a fraction of the protein mixture which was no longer hydrophilic. Denaturation or polymerization could be responsible for the rise in lipophilicity.

The human IgG showed 35-40% non-specific binding when 1 mg/ml protein is incubated with vesicles that did not have a protein binding

Table 3-1. Specific and Non-Specific Binding of Antibodies to Vesicles.

Antibody (1 mg/ml)	<u>Binding</u>	
	Non-Specific	Specific
Anti-Breast Cancer Monoclonal Antibody	14.6	29.3
Anti-Prostatic Acid Phosphatase Monoclonal Antibody	6.7	30.9
Anti-Horseradish Peroxidase Monoclonal Antibody	0.5	13.0
Human IgG Polyclonal Antibody	31.8	45.9

agent. When the unbound protein fraction from this preparation was reincubated with similar vesicles, the non-specific binding dropped to 10-13%. A further incubation of unbound protein with vesicles had binding too low to accurately measure, but was significantly below 5%. This is a potentially useful procedure that removes denatured and polymerized antibody from the otherwise good protein.

After determining that the non-specific binding was relatively constant, the analysis of the various protein modification agents was undertaken. Two concentrations of protein were used, and all experiments were done with triplicate analyses to be sure of reproducibility. The vesicles were prepared with the cholesterol derivative and DSPC in the same manner as Baldeschwieler et al.⁴ The lipids were made into vesicles using an ultrasonic probe. After annealing and centrifuging the vesicles were incubated with the iodinated human IgG. The resulting mixture was passed down preconditioned Sepharose 4B columns and fractions collected. The vesicles eluted first, with the protein eluting later. The various fractions were counted and the binding efficiency calculated. The vesicle fractions were stored for 24 hours and passed through the columns again. The amount of activity that stayed with the vesicle fraction was a direct measure of the stability of the antibody-vesicle complex.

The results are shown in Table 3-2. A ten-fold difference in protein concentration made a significant variation in binding efficiency. This was probably due to a kinetic effect, as there were approximately equivalent numbers of vesicles and antibody molecules at the 100 µg/ml concentration; there was on the order of 10 times the

Table 3-2. Protein Binding by 8:1 DSPC:Cholesterol Derivative Vesicles at 1 Hour Incubation.

Binding Agent	1 mg/ml Binding	100 μg/ml	
		Binding	Stability
V	40.3%	77.5%	94.1%
IX	46.0%	69.5%	91.0%
XIII	43.3%	60.4%	91.8%
XVI	31.9%	55.9%	86.5%
XVII	39.5%	61.1%	86.0%
III (non-specific)	31.9%	58.7%	86.4%

number of antibody molecules as vesicles at the 1 mg/ml antibody concentration. The protein modification agents, especially the N-hydroxysuccinimide esters, have a limited half-life in the basic saline and will not have a chance to interact with as high a percentage of the antibody molecules at the higher concentration. The 24 hour stability was generally good for all of the cholesterol derivatives (even for non-specific) the greatest stability was found for the better binding agents. This may be due to the antibodies actually stabilizing the vesicle. The better cholesteryl derivatives bound more antibody, leading to a greater stabilizing effect.

The results were ambiguous in the determination as to which of the cholesterol derivatives was the best binding agent. Both cholesteryl 6-carboxyhexyl ether N-hydroxysuccinimide ester and cholesteryl 5-carboxypentyl ether N-hydroxysuccinimide ester performed well. The other agents did not bind protein as effectively, and the cholesteryl 6-isothiocyanatohexyl ether was no higher than non-specific binding. It presumably was so lipophilic that the NCS group stayed within the phospholipid bilayer and never got into the aqueous phase to interact with the protein. There was a general correlation between hydrophilicity of the binding groups and protein binding efficiency.

The viability of the bound antibody was determined using a radioimmunoassay (RIA). A number of difficulties arose in trying to adapt standard RIA techniques to the vesicle-antibody system. In the usual antibody RIA, a standard curve of various known amounts of antibody are analyzed in a similar way as the sample. The antibody is incubated with iodinated antigen, forming antibody-antigen polymers. This either precipitates on its own or is precipitated by the addition

of a second antibody (an anti-antibody). The amount of radioactivity deposited on the tubes is a direct measure of the amount of antibody present in the original solution. The value for the sample is compared with the standard curve to find the concentration of antibody.

This technique was not useful with the vesicle-antibody complex. The vesicle dominated the solubility of the complex, and precipitation by the second antibody is much less than it was for the unbound antibody. This complication necessitated two more sets of standards. These were to determine the precipitation rate of both the free antibody and the vesicle-antibody complex. This was accomplished using iodinated antibody. The iodinated antigen was not added, since two iodinated species in solution would further complicate the situation. Dilutions of the iodinated antibody were prepared identically to those of the uniodinated standards. Since all antibodies have some affinity for glass, and a serial dilution was performed, a significant error in antibody concentration had developed for the more dilute concentrations. The iodinated antibody standards were used to determine the actual concentration of antibody in each of the uniodinated standards. After incubation with antigen (for the standards), the second antibody was added to each of the standards. The tubes were incubated for 10 minutes, centrifuged and emptied. The counts in the iodinated antibody standard tubes were a direct measure of the precipitation rate. The binding in the standards were then adjusted to reflect the precipitation rate.

The vesicles were handled in a similar fashion. Two identical vesicle preparations were incubated with antibody solutions, one of which was iodinated. The iodinated antibody determined the percent

binding of antibody to vesicles. This was used as the total bound antibody figure used in the final calculation. Identical samples of both the iodinated and the uniodinated antibody-vesicle complex were taken and the uniodinated complex incubated with iodinated antigen. A different second antibody was used to precipitate the vesicles, since it was found to be more effective for vesicles, and the other second antibody was more effective for the free antibody. The binding of iodinated antigen was determined and corrected for the precipitation rate, which was determined using the iodinated vesicle-antibody complex.

The corrected sample was compared with the corrected standard curve. This gives a value for total viable antibody on a vesicle sample. The result is divided by the total bound antibody value determined earlier. The percent active antibody on the vesicles was 40 percent.

The 40 percent figure is reasonable because the protein modification agents will bind at essentially random sites on the antibody. If it binds at a site which makes the antigen specific ends point toward the vesicle, then the antigen cannot bind with the antibody. There is approximately a 50 percent chance that the antigen binding sites will be blocked by the vesicle. The fact that 40 percent were still viable shows that the protein binding agent did not denature the antibody to any great extent.

The in vitro cell assay which is described in the next chapter confirmed the RIA results. Both assay techniques determined that at least one viable antibody was present on every vesicle. The sample calculation in Figure 3-1 gives an indication of antibody-vesicle-technetium stoichiometry in these very dilute systems.

The technetium complex $[\text{Tc}(\text{CN}-\text{I})_6]^+$ labels vesicles to a reasonable extent. When it is formed 30-35% of the isonitrile complex ends up bound to the glass walls of the kit reaction vial. Between 30% and 45% of this activity can be transferred to the vesicles by treating the "empty" vial with a saline solution of vesicles (e.g. 2.0 ml of vesicles in saline ca. 10^{13} - 10^{14} vesicles with 6-7 mCi of $^{99\text{m}}\text{Tc}$). Thus, the final yield of $^{99\text{m}}\text{Tc}$ bound to the vesicles is of the order of 10-15%. Unlike the free technetium isonitrile complex the vesicle-bound species is not "sticky" with respect to glass and metal surfaces to any great extent. The mode of binding of the isonitrile complex to the vesicles is most probably the non-specific attachment of the $[\text{Tc}(\text{CN}-\text{I})_6]\text{Cl}$ ion pair to the phospholipid bilayer. The complex is lipophilic and has a high affinity for hydrophobic surfaces such as glass, vesicles and cells.

CONCLUSION

The work discussed in this chapter shows that a number of cholesteryl ethers stabilize vesicles and conjugate antibodies to the vesicle surface. The two best are cholesteryl 6-carboxyhexyl ether N-hydroxysuccinimide ester and cholesteryl 5-carboxypentyl ether N-hydroxysuccinimide ester. Once bound the antibodies remain viable to an extent of about 40% and that the vesicles could be labelled by a technetium-99m complex.

Figure 3-1

Sample Calculation of Antibody and
Technetium Binding to Vesicles

Vesicles:

Total Weight: 6 mg lipid layer, 6 mg aqueous layer = 12 mg total
weight

Each Vesicle 700×10^{-10} m diameter $V = 4/3\pi r^3$

$V = 1.80 \times 10^{-22} \text{ m}^3$

Density = 1 g/ml = 1000 kg/m³

Weight/Vesicle = 1.80×10^{-19} Kg

$$\frac{\text{Initial Weight}}{\text{Weight/Vesicle}} = \frac{1.2 \times 10^{-5} \text{ Kg}}{1.80 \times 10^{-19} \text{ Kg/vesicle}} = 6.68 \times 10^{13} \text{ Vesicles}$$

Antibody:

Molecular Weight 150,000 g/mole

0.50 ml of 1 mg/ml solution = 500 μ g protein

$$\frac{(500 \mu\text{g})(1 \times 10^{-6} \text{ g}/\mu\text{g})}{150,000 \text{ g/mole}} = 3.33 \times 10^{-9} \text{ mole}$$

6.02×10^{23} molecules/mole

2.01×10^{15} molecules IgG added

	Total Molecules	Molecules/Vesicle
Total Antibodies Added	2.01×10^{15}	30
Total Bound Antibody (30% of total added)	6.03×10^{14}	9
Viable Antibody on Vesicles (40% of bound)	2.41×10^{14}	3.6

Figure 3-1 (continued)

Technetium:

1×10^{-6} molar $^{99m}\text{TcO}_4^-$ Generator Eluant

1 ml = 1×10^{-9} moles Tc-99m

15% bind to vesicles = 1.5×10^{-10} moles Tc-99m

6.02×10^{23} molecules/mole

9.03×10^{13} molecules Tc-99m

with 6.68×10^{13} vesicles

1.4 molecules Tc-99m/Vesicle

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CHAPTER IV

ANALYSIS OF THE VESICLE-ANTIBODY COMPLEX IN LIVING SYSTEMS

INTRODUCTION

The work described in this chapter details: (i) the in vitro cell bound antigen assay of the labelled vesicle bound antibodies, (ii) the imaging and biological distribution of the labelled vesicle-antibody complex in sub-cutaneous tumor-bearing mice, and (iii) the imaging and biological distribution of the labelled vesicle-antibody complex in subrenal tumor-bearing mice.

The work in this chapter could never have been completed without the help of a number of people at New England Nuclear Corp. The monoclonal antibodies, the cell lines, laboratory space, and invaluable assistance were provided as gifts, and many evenings and weekends were sacrificed by these people so that the experiments could be completed. Thanks are owed to William Neacy, Richard Harrison, Peter Jones, Brian Gallagher, and Howard Sands, among others.

EXPERIMENTAL

In Vitro Cell Assay of Antibodies Bound to Vesicles

A monoclonal IgG antibody directed against the Thy1.1 antigen⁴ was iodinated with ¹³¹I using the Iodogen method described in the previous chapter.² A control monoclonal IgG antibody directed against prostatic acid phosphatase was iodinated by the same method with ¹²⁵I. The specific activity of each of these antibodies was on the order of 1 mCi/mg.

Each of the antibodies was conjugated to a preparation of vesicles using the technique outlined earlier, and the vesicle-antibody complex was separated from the unbound antibody using size exclusion gel chromatography (Sephacrose 4B, 1 x 15 cm column, eluant PBS). The final solutions contained about 6 mg of lipid as vesicles and about 200 micrograms of antibody bound to vesicles.

The vesicle preparations were labelled with ^{99m}Tc following the procedure outlined in the previous chapter. In short, ^{99m}TcO₄⁻ is reacted to form hexakis-t-butyl isonitrile technetium (I) cation. The vesicle preparations were shaken for 15 minutes in the reaction vial. Typically about 6 mCi of ^{99m}Tc would be bound to each of the vesicle preparations. These were then diluted so that the protein concentration was 100 ng/0.1 ml; usually this involved a 1:50 dilution. It was important that all the antibody solutions have the same protein content, otherwise valid comparisons could not be made between preparations. A sample of unbound specific antibody (with no vesicles) was also diluted to 100 ng/0.1 ml. This was included as a reference for the vesicle-bound antibody binding.

Two tumorigenic lymphocytic cell lines were grown in culture, a Thy1.1+ line (SL2) and a Thy1.1-line (SL1).¹ The cells from each cell line were serially diluted using 1% bovine serum albumin (BSA) in RPMI-1641 buffer.³ Typical dilutions produced cell concentrations of 1×10^7 , 5×10^6 , 1×10^6 , 5×10^5 , 1×10^5 , and 5×10^4 cells/0.2 ml for each of the cell lines.

Six separate series of cell assay were performed: (i) the unbound specific antibody (anti Thy1.1) with the specific cell line (SL2), (ii) the unbound specific antibody with the control cell line (SL1), (iii) the specific antibody bound to vesicles with the specific cell line, (iv) the specific antibody bound to vesicles with the control cell line, (v) the control antibody (anti PAP) bound to vesicles with the specific cell line, and (vi) the control antibody bound to vesicles with the control cell line.

Duplicate samples (0.2 ml) of the two cell lines at each of the six concentration levels were placed in three sets of twenty-four tubes. A sample (0.1 ml) of the appropriate antibody preparation (unbound specific antibody, vesicle bound specific antibody, and vesicle bound control antibody) was added to each tube (one antibody preparation per set of twenty-four tubes). All the tubes were mixed by agitation and allowed to stand at room temperature for one hour. The first four tubes from each set were counted for ^{125}I , ^{131}I , and $^{99\text{m}}\text{Tc}$ activity to determine the total counts added to each set of tubes. All the tubes were centrifuged at 400xg for two minutes to pelletize the cells. The supernatant was removed by aspiration and buffer (0.3 ml) was added to each tube. The cells were resuspended and recentrifuged (400xg, 2 minutes). The supernatant was removed and the cells were

washed with buffer a second time as above. The cells were counted for each of the three nuclides. The activity in each tube was divided by the total counts added to determine the percentage binding of the radiolabel.

The Imaging of Sub-Cutaneous Tumors with Technetium-99m Labelled Vesicle-Antibody Complexes

The animals used were AKR/Cumberland mice, 4 to 6 weeks old, obtained from Cumberland View Farms, Clinton, Tennessee. Each of the mice was injected in their right thigh sub-cutaneously with 1×10^7 SL2 cells in 0.1 ml. The tumors were allowed to grow for 14 days. The tumors grew to be large and slightly impaired the movement of the mice. The specific monoclonal antibody (anti Thy1.1) was iodinated with ^{131}I as described earlier. The control antibody (anti PAP) was iodinated by the same method with ^{125}I . The antibodies were coupled to two vesicle preparations, and the vesicle-antibody complex was separated from the unbound antibody using size exclusion gel chromatography (Sephacrose 4B, 1 x 15 cm column, eluant PBS) as described in the previous chapter. The final solutions contained about 6 mg of lipid as vesicles and 0.200 mg protein conjugated to the vesicles.

The vesicles were labelled with $^{99\text{m}}\text{Tc}$ as described earlier. The two preparations were added together, diluted with saline, and injected into the tail vein of the tumor-bearing mice. Injection standards were taken to determine the injected dose for each of the three nuclides. After three hours, the mice were imaged using a Med-X modified Pho/Gamma HP camera (Nuclear-Chicago) fitted with a 5 mm pinhole collimator. Mice were positioned with their ventral surface up. Each image represents 50,000 counts accumulated.

After six hours, the imaging was repeated, and the two mice with the best tumor images were sacrificed. These mice were dissected and their organs counted separately. Twelve organs were collected per mouse, including the tumors (blood, tumor, liver, spleen, kidneys, muscle, bone, stomach, upper g.i., lower g.i., heart, and lungs). The tumors were, for the most part, very large (about 1 g) and necrotic. At 24 hours post injection, the remaining mice were sacrificed and dissected to determine the biological distribution of the three labels. The organs were counted for the various nuclides. The results from the gamma counter were corrected for cross counts from one nuclide into another nuclide's channel and for radioactive decay. The data were calculated for both percentage of injected dose per organ and percentage of injected dose per gram of organ.

The Imaging of Subrenal Tumors with the Vesicle-Antibody Complex

A tumor was grown in a normal AKR/Cumberland mouse. The mouse was injected sub-cutaneously with 1×10^7 SL2 cells in 0.1 ml, and the tumor was allowed to grow for 12 days (to about 0.5 g). It was then removed and cut into approximately 1 mg pieces. Normal AKR/Cumberland male, 6 to 8 week-old mice were then used for the tumor implantation.

The mice were anesthetized using sodium pentobarbital, and tumors were implanted in the subrenal capsule as follows. An incision was made in the skin of the lower left back of the mouse, and the peritoneum was opened to expose the left kidney. The kidney was then isolated from the other organs using a curved hemostat, and a trocar was used to implant the tumor pieces as follows. (A trocar is a device consisting of a wide bore needle with a moveable center rod.) A small

shallow incision was made at the lower end of the kidney, cutting the renal capsule. (The renal capsule is a membrane that surrounds the kidney.) The forward edge of the renal capsule was grasped with small forceps and the trocar inserted between the kidney and capsule. The tumor was pushed out of the trocar using the central rod and held in place using the back of the forceps. The trocar was removed and the tumor pushed further up under the capsule. The capsule was smoothed and checked for breaks. The kidney was then replaced inside the peritoneal cavity and the peritoneum closed. The opening in the skin was closed using one or two wound clips. The mice were allowed to recover from the anesthesia and checked for bleeding or other complications. The entire surgery usually took less than five minutes of open wound time.

The tumors were allowed to grow and vascularize for 9 days, to about 100 mg in size. The two antibodies were iodinated (^{131}I on anti-Thy1.1 and ^{125}I on anti-PAP) and conjugated to vesicles as described in the previous chapter. The vesicle preparations were separately labelled with $^{99\text{m}}\text{Tc}$ in the usual way.

Samples of the vesicle preparations were combined and diluted with saline so that each iodine isotope was about 3.5 mCi/0.1 ml. Since the isotopes were originally about 140 mCi/ml, about a 1 part specific antibody vesicles:1 part non-specific antibody vesicles:2 parts saline dilution was used. Tumor-bearing mice to be used for the biological distribution studies were injected with 0.1 ml of this vesicle mixture. Tumor-bearing mice to be used for imaging on the gamma camera were injected with 0.1 ml of the pure specific antibody vesicle preparation. This was about 217 microcuries of $^{99\text{m}}\text{Tc}$ and 13 microcuries of ^{131}I per mouse.

At three, six and eight hours post injection, the image mice were anesthetized and imaged for ^{99m}Tc . The camera was set up the same as in the sub-cutaneous study. The mice were positioned with the dorsal surface up. Each ^{99m}Tc image represents 100,000 counts accumulated. At eight hours post injection, the image mice were also imaged for ^{131}I activity using the same configuration but a different energy setting for the gamma camera. Each ^{131}I image represents 25,000 counts accumulated. A group of the biological distribution mice was sacrificed and dissected at both the three and six hour time points. Ten organs were collected per mouse, including tumor and the uninvolved right kidney (blood, tumor, liver, spleen, right kidney, muscle, bone, stomach, upper g.i., and lower g.i.). The whole mouse and the individual organs were all weighed.

At 23 hours post injection, the image animals were imaged for both ^{99m}Tc and ^{131}I activity. By this time the ^{99m}Tc had decayed a significant amount and long counting times (approximately 30 minutes) were needed for the images. At 24 hours post injection a third group of the biological distribution mice was sacrificed and dissected.

At 48 hours post injection, the final group of biological distribution mice were sacrificed and dissected. The image mice were also sacrificed and dissected in an identical procedure as the biological distribution mice. All of the samples from the various mice were counted on the gamma counter for ^{125}I , ^{131}I and ^{99m}Tc activity. The results from the gamma counter were corrected for cross counts from one nuclide into another nuclide's channel and for radioactive decay. The results were calculated for both percentage of injected dose per organ and percentage of injected dose per gram of organ.

RESULTS AND DISCUSSION

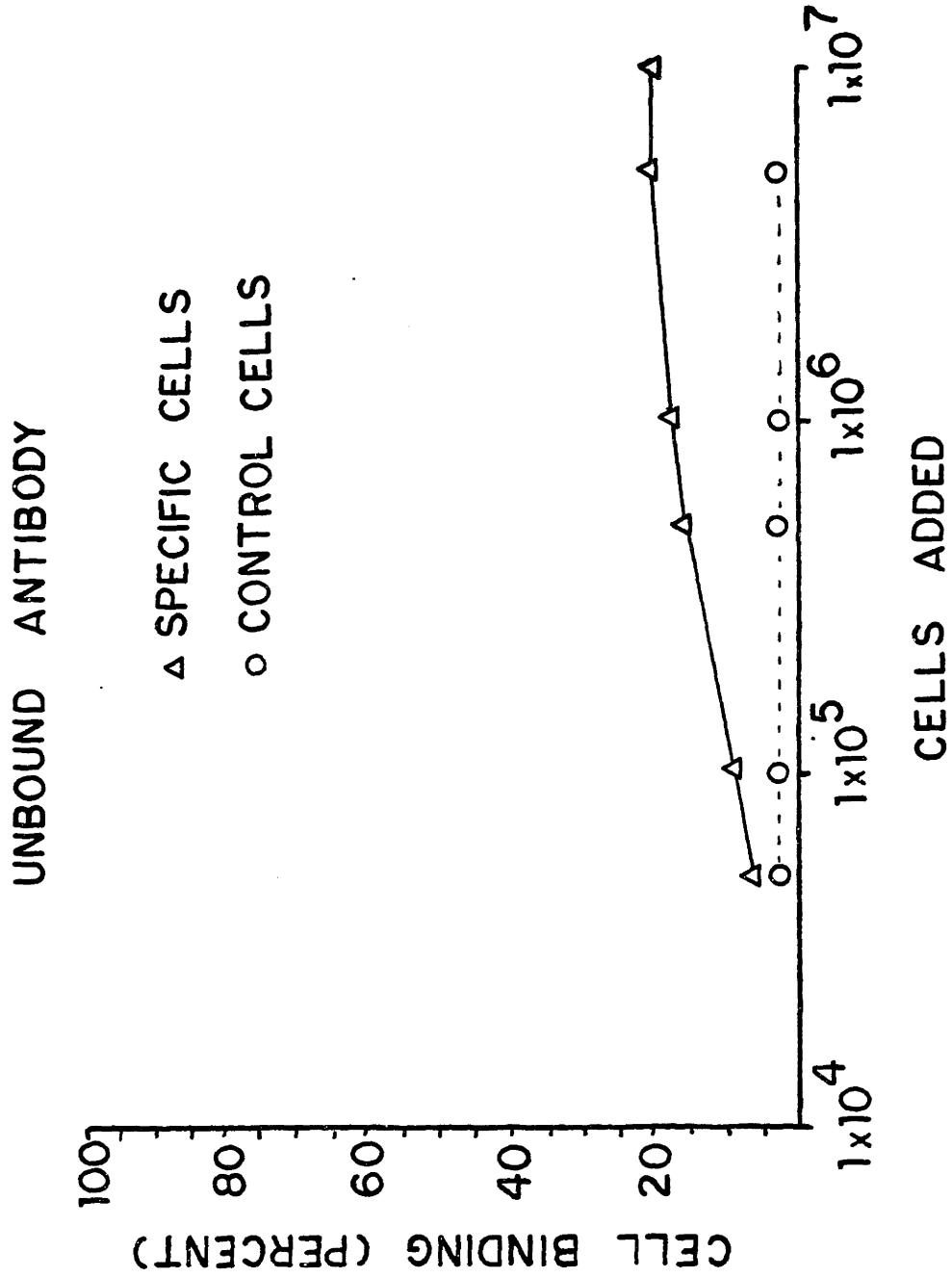
The vesicle-antibody complex was analyzed using living systems; cells in vitro and tumors in vivo. AKR/Cumberland mice were used for all the studies because they did not express the Thy1.1 antigen on their lymphocytes.¹ The antibody used was specific for the Thy1.1 antigen, and the goal was to have a tumor specific antigen-antibody reaction. If there had been circulating antigen, then the specific antibody on the vesicles would have had a high affinity for the lymphocytes as well as the tumor, lowering the specificity for tumor dramatically. The presence of tumor-specific antigens in spontaneous cancers has been debated, but the antigen is definitely tumor-specific in the model used in this study.

A cell assay of the vesicle-antibody complex was performed with a double control. The specific antibody coupled to vesicles was incubated with both antigen positive and antigen negative lymphoma cells. The control antibody was also incubated with antigen positive and antigen negative cells. This double control assured that the specificity measured was not due to an artifact of the experimental design or the characteristics of the vesicle-antibody complex. Specific antibody which was not bound to vesicles was also used as a further control for both the cells and the antibody viability.

The results from the unbound antibody cell assay are shown in Figure 4-1. There was clearly a specificity of the antibody for the antigen positive cells. Other cell assays of this antibody showed higher binding,⁶ and the reason for a maximum binding of only 20% is not known. This could have been due to some of the antigen positive cells changing to or being contaminated with antigen negative cells. The

Figure 4-1

Unbound Specific Antibody Cell Assay.



control antigen negative cells showed very low binding, which confirmed the nature of these cells.

The vesicle bound iodinated antibody cell assay showed the expected specificity (Figure 4-2). The nuclides counted were ^{125}I and ^{131}I , which were on the antibodies, not the vesicles. Only the specific antibody with the specific cells showed significant binding. This was a dramatic confirmation of the RIA described in the previous chapter. The cell binding was much higher than that of the vesicle free antibody. This can be interpreted to mean that there was either denatured protein in the vesicle free antibody, or that more than one viable antibody was bound to each vesicle. In either event, the vesicle bound antibody showed very high specificity and binding, giving clear evidence that the concept of antibody directed vesicle distribution has merit.

When the same assay was counted for $^{99\text{m}}\text{Tc}$, however, very different results were obtained (Figure 4-3). The vesicles had been labelled with the isonitrile technetium complex, and it appears that the labelling was reversible. All of the various antibody and cell combinations showed essentially the same binding. Since the same vesicles when counted for iodine showed good specificity, the specific vesicles were not bound to the control cells, nor were the control antibody vesicles bound to either of the cell lines. The technetium binding seemed to be completely independent of this binding, and therefore was independent of the vesicles. The isonitrile complex has an undeniable affinity for vesicles, but seems to have a greater affinity for cells, leaving the vesicles and binding to the cells regardless of any antibody specificity.

Figure 4-2

Vesicle Bound Iodinated Antibody Cell Assay.

ANTIBODY BOUND TO VESICLES

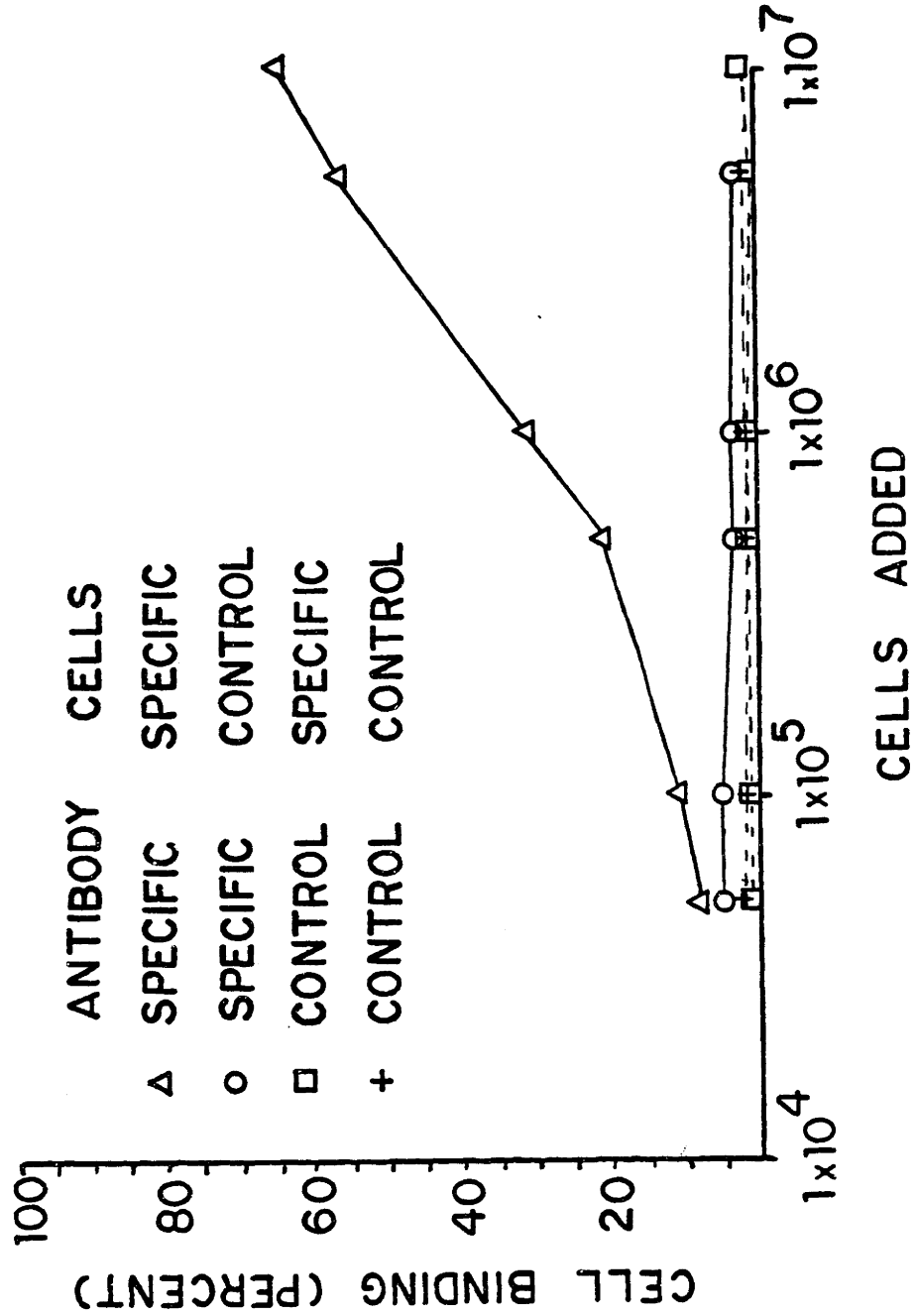
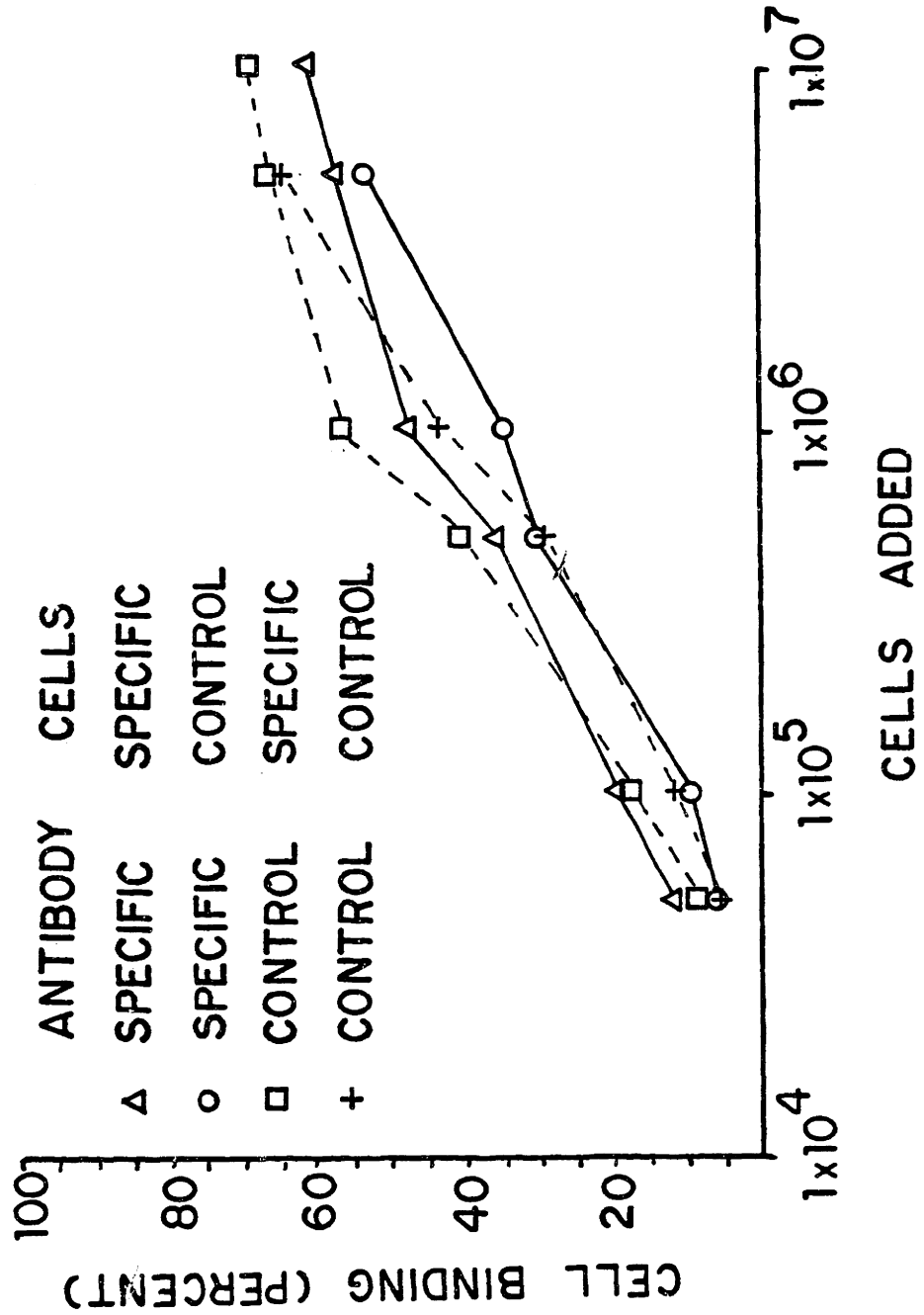


Figure 4-3

Technetium Labelled Vesicles Cell Assay.

TECHNETIUM "BOUND" TO VESICLES

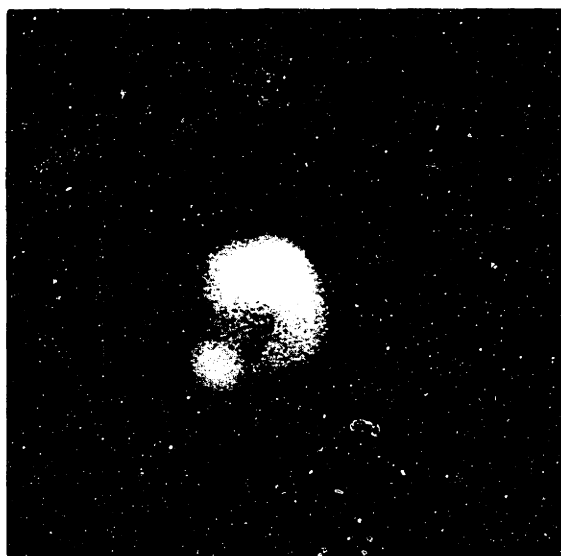


The cell assay results clearly indicate that a different vesicle labelling procedure needs to be developed. In spite of the fact that the isonitrile technetium complex labelled the vesicles well, the label was labile and did not stay with the vesicles when exposed to a cell membrane. Some type of covalent attachment of technetium to the vesicle would be preferred. Perhaps a chelate could be bound to a cholesterol derivative, which could sequester technetium. Alternatively, iodine could be bound to a cholesterol derivative, and this would become part of the structure during the formation of the vesicles. A number of other approaches could also be used to label vesicles with a radionuclide. Whatever labelling procedure is developed, it is clear that an approach different from that used in this study needs to be taken.

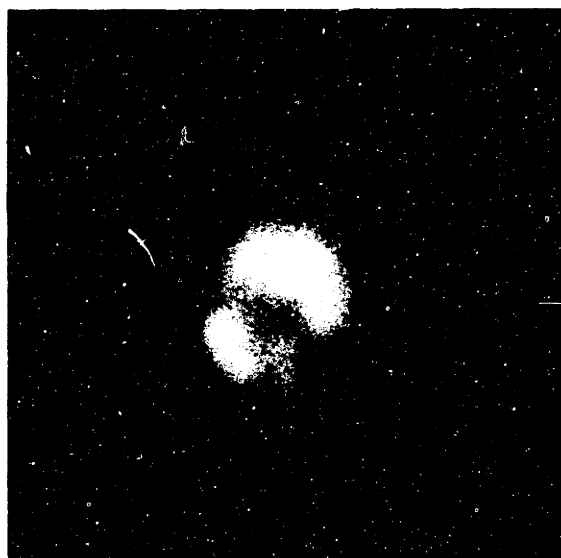
In spite of the obvious lack of a dependable technetium label for the vesicles, tumor imaging was attempted to determine whether or not the antibody would direct the vesicles in vivo. Sub-cutaneous tumors were grown in mice and injected with both the specific and control antibodies bound to vesicles. Gamma camera images of the ^{99m}Tc distribution were taken at 3 and 6 hours post injection (Figure 4-4) and show liver, spleen, kidneys, and intestines. The images seem to show tumor to a reasonable extent, but the biological distribution of the activity does not confirm this (Figure 4-5). Perhaps the "tumor" image was really the left kidney, which was close to the tumor site. Kidneys were among the most heavily labelled organs. The tumor contained very little ^{99m}Tc activity, and the iodine activity from both antibodies was equally low.

Figure 4-4

Gamma Camera Images of Sub-Cutaneous Tumor Bearing Mice.
The Images are of the Tc-99m Distribution.



2.5 HOURS POST INJECTION



6.0 HOURS POST INJECTION

Figure 4-5

Biological Distribution of ^{125}I , ^{131}I and $^{99\text{m}}\text{Tc}$ in
Sub-Cutaneous Tumor Bearing Mice.

ORGAN	CONTROL ANTIBODY		SUB-CUTANEOUS TUMOR BIODISTRIBUTION		SPECIFIC ANTIBODY		TECHNETIUM LABEL	
	%i.d./gram	%i.d./organ	%i.d./gram	%i.d./organ	%i.d./gram	%i.d./organ	%i.d./gram	%i.d./organ
SEVEN HOURS POST INJECTION								
BLOOD	1.62+/-0.94	1.76+/-1.03	1.65+/-0.80	1.79+/-0.88	0.27+/-0.01	0.30+/-0.01	0.23	0.11
TUMOR	0.60	0.33	0.65	0.23	0.23	0.02	0.30	4.14
LIVER	5.66	6.29	11.61	7.46	14.63	9.84	1.02	0.10
SPLEEN	5.51	7.17	3.60	4.26	0.35	0.42	4.46	1.52
KIDNEYS	1.11	0.68	1.76	1.05	0.60	0.35	0.42	3.62
MUSCLE	0.29	0.19	0.31	0.14	2.72	1.20	0.34	0.73
BONE	0.60	0.34	0.57	0.23	1.24	0.51	2.21	0.60
STOMACH	15.13	10.34	18.08	9.30	5.10	3.29	3.82	5.30
UPPER G.I.	0.54	0.34	1.39	0.17	1.92	0.18	4.03	5.18
LOWER G.I.	4.43	4.62	12.12	6.60	15.57	8.50	1.32	0.21
HEART	0.79	0.41	0.82	0.23	0.13	0.05	0.34	0.08
LUNGS	0.89	1.15	0.82	1.01	0.18	0.11		
TWENTY-FOUR HOURS POST INJECTION								
BLOOD	0.47	0.05	0.27	0.04	0.33	0.01	0.13	0.16
TUMOR	0.20	0.04	0.22	0.06	0.16	0.02	0.20	0.15
LIVER	1.84	0.18	2.29	0.21	3.53	0.10	6.69	10.28
SPLEEN	3.86	0.50	2.53	0.15	0.24	0.02	1.12	0.11
KIDNEYS	0.33	0.09	0.44	0.09	0.16	0.01	7.91	3.01
MUSCLE	0.07	0.02	0.05	0.02	0.53	0.21	0.70	6.70
BONE	0.14	0.02	0.13	0.03	0.31	0.09	0.38	0.92
STOMACH	1.17	0.36	0.57	0.21	0.26	0.14	0.93	0.39
UPPER G.I.	0.17	0.06	0.12	0.04	0.21	0.05	1.00	1.79
LOWER G.I.	0.18	0.07	0.18	0.07	0.19	0.08	1.49	1.60
HEART	0.18	0.05	0.12	0.02	0.02	0.00	2.00	0.29
LUNGS	0.35	0.08	0.27	0.09	0.06	0.02	0.70	0.15

Another set of animals was sacrificed at 24 hours post injection and the tumor was still not heavily labelled (Figure 4-5). This also showed that there was no significant localization of the specific antibody in the tumor. The ratio of specific antibody to control antibody in the tumor was essentially the same as the ratio of specific to control antibody in the muscle. Since there was no specific localization, the activity in the tumor was probably due to passive factors such as blood flow and vascular permeability.

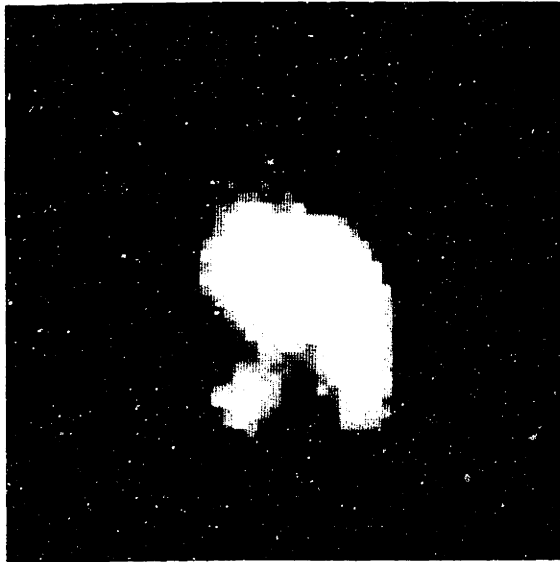
The lack of labelling in the sub-cutaneous tumors might have been due to the label not reaching the tumor because of low blood flow. Since the blood flow is much better in the kidney than sub-cutaneously, tumors were implanted in the subrenal capsule. Small (1 mg) pieces of tumor which had been grown sub-cutaneously were implanted in the mice. The tumors were allowed to grow to about 100 mg (for 9 days).

Two sets of tumor-bearing mice were injected. One set, which was used for the biological distribution studies, was injected with both the specific antibody on vesicles and the non-specific antibody on vesicles. Since the two antibodies were labelled with different iodine isotopes, the distribution of the two labels could be determined independently. The distribution of the ^{99m}Tc on both sets of vesicles was also followed, in spite of the fact that it probably was not on the vesicles in vivo. The other set of mice were injected with only the specific antibody on vesicles, at a higher concentration, so the distribution could be followed by gamma camera images.

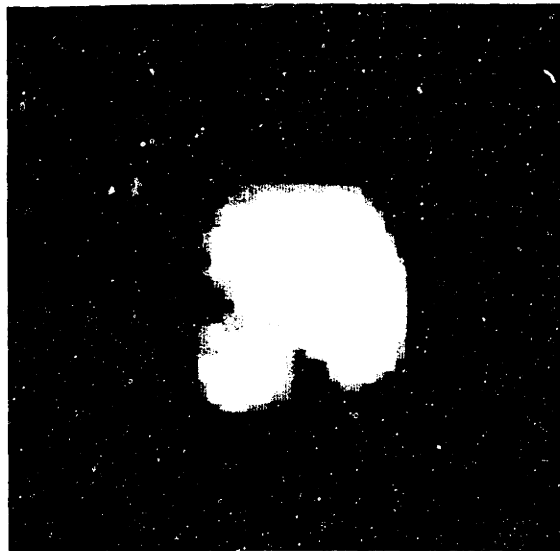
Technetium images were taken at 3, 6, 8, and 23 hours post injection; ^{131}I images were taken at 8 and 23 hours post injection. The images are shown in Figure 4-6. The technetium images show the expected

Figure 4-6

Gamma Camera Images of Subrenal Tumor Bearing Mice.
The Images at 3.0 and 6.0 Hours Post Injection are Tc-99m
Images. The Others are I-131 and Tc-99m Images.



3.0 HOURS POST INJECTION



6.0 HOURS POST INJECTION

I-131

Tc-99m



8 HOURS POST INJECTION



23 HOURS POST INJECTION

liver, spleen, intestinal, and kidney activity. These are the organs in which the unbound isonitrile complex localizes. It was difficult to tell if there was any specific uptake in the tumor. The right kidney was obscured by liver, spleen, and intestines, so the kidney activity was difficult to judge. The iodine images were unambiguous. The eight hour image showed thyroid, tumor, and bladder; the 23 hour image just showed thyroid and tumor. This was very exciting, but the biological distribution data failed to support this observation (Figure 4-7). The tumor activity for both iodine and technetium was very low at every time point.

The left kidney was not counted for the biological distributions; only the right kidney was used. This was because the tumor/non-tumor separation was not always easily distinguished, so considerable damage was usually done to the left kidney while isolating the tumor. It was felt that the right kidney was a reasonable control for kidney localization. In retrospect, the activity seen in the images must have been localized somewhere, and it was not in the tumor in the biological distribution mice. There are at least two and probably more explanations for these observations. One is that since the imaged mice received a larger dose of the specific antibody, it stayed localized in those tumors longer than it remained in the tumors of the mice used in the biological distribution studies. If the maximum localization occurs at some point between six and twenty-four hours, the images will be good at eight hours, but the localization will not be seen in the biological distributions. Another explanation is that the tumor, while growing in the subrenal capsule, has damaged the kidney in such a way as to increase its affinity for vesicles or the antibody. The images were simply showing left kidney (exclusive of tumor), which was not counted.

Figure 4-7

Biological Distribution of ^{125}I , ^{131}I , and $^{99\text{m}}\text{Tc}$ in
Subrenal Tumor Bearing Mice.

SUBRENAL TUMOR BIODISTRIBUTION

ORGAN	CONTROL ANTIBODY		SPECIFIC ANTIBODY		TECHNETIUM LABEL	
	%i.d./gram	%i.d./organ	%i.d./gram	%i.d./organ	%i.d./gram	%i.d./organ
THREE HOURS POST INJECTION						
BLOOD	0.73+/-0.06	0.98+/-0.13	1.00+/-0.09	1.34+/-0.16	0.13+/-0.03	0.18+/-0.02
TUMOR	0.33	0.04	0.42	0.05	0.62	0.08
LIVER	0.25	0.39	0.38	0.60	3.81	5.88
SPLEEN	0.39	0.04	0.47	0.05	1.01	0.10
RT. KIDNEY	0.45	0.10	0.67	0.14	2.52	0.53
MUSCLE	0.13	1.35	0.15	1.61	0.13	1.39
BONE	0.28	0.76	0.35	0.92	0.10	0.28
STOMACH	3.30	1.21	3.99	1.46	0.77	0.28
UPPER G.I.	0.32	0.41	0.41	0.54	1.39	1.67
LOWER G.I.	0.30	0.30	0.41	0.43	1.71	1.74
SIX HOURS POST INJECTION						
BLOOD	2.51	1.73	2.75	3.33	0.20	0.12
TUMOR	1.03	0.71	1.16	0.13	1.52	0.18
LIVER	3.49	4.38	3.08	3.21	5.75	6.14
SPLEEN	7.82	9.50	4.14	0.35	1.54	0.14
RT. KIDNEY	1.48	0.86	1.85	0.37	7.21	1.44
MUSCLE	0.56	0.41	0.55	5.25	0.46	4.29
BONE	0.77	0.45	0.84	2.02	0.38	0.90
STOMACH	13.12	5.28	13.66	3.71	1.33	0.35
UPPER G.I.	1.62	1.21	1.73	2.04	1.66	2.01
LOWER G.I.	1.09	0.60	1.44	1.66	3.16	3.39
TWENTY-FOUR HOURS POST INJECTION						
BLOOD	0.53	0.14	0.68	0.84	0.05	0.06
TUMOR	0.17	0.05	0.32	0.03	2.40	0.21
LIVER	0.13	0.04	0.22	0.33	3.10	4.70
SPLEEN	0.17	0.05	0.20	0.02	0.65	0.06
RT. KIDNEY	0.35	0.10	0.55	0.11	8.82	1.84
MUSCLE	0.05	0.01	0.06	0.56	0.34	3.41
BONE	0.08	0.04	0.11	0.27	0.26	0.65
STOMACH	1.10	0.45	1.00	0.71	0.49	0.35
UPPER G.I.	0.19	0.08	0.20	0.31	0.73	1.13
LOWER G.I.	0.16	0.05	0.20	0.21	0.58	0.60

SUBRENAL TUMOR BIODISTRIBUTION (CONTINUED)

ORGAN	CONTROL ANTIBODY		SPECIFIC ANTIBODY		TECHNETIUM LABEL	
	%i.d./gram	%i.d./organ	%i.d./gram	%i.d./organ	%i.d./gram	%i.d./organ
FORTY-EIGHT HOURS POST INJECTION						
BLOOD	0.21+/-0.10	0.29+/-0.15	0.34+/-0.21	0.48+/-0.30	0.15+/-0.08	0.21+/-0.11
TUMOR	0.15 0.03	0.02 0.01	0.28 0.07	0.04 0.02	3.23 1.28	0.46 0.12
LIVER	0.44 0.28	0.77 0.52	0.59 0.37	1.04 0.68	2.67 0.28	4.65 0.83
SPLEEN	1.90 1.63	0.22 0.19	1.02 0.81	0.12 0.09	1.33 0.79	0.15 0.10
RT. KIDNEY	0.42 0.06	0.09 0.02	0.54 0.13	0.11 0.02	10.15 1.00	2.12 0.36
MUSCLE	0.02 0.01	0.25 0.08	0.04 0.01	0.44 0.17	0.68 0.16	7.72 1.79
BONE	0.04 0.02	0.11 0.04	0.09 0.04	0.24 0.11	0.36 0.13	1.01 0.38
STOMACH	0.30 0.28	0.16 0.18	0.28 0.26	0.15 0.16	0.69 0.37	0.36 0.08
UPPER G.I.	0.08 0.04	0.12 0.05	0.09 0.04	0.13 0.06	0.51 0.05	0.79 0.11
LOWER G.I.	0.07 0.03	0.08 0.02	0.09 0.04	0.10 0.03	0.42 0.03	0.50 0.14

IMAGE ANIMALS FORTY-EIGHT HOURS POST INJECTION

BLOOD	--	--	0.24	0.10	0.31	0.14	0.13	0.05	0.17	0.07
TUMOR	--	--	0.16	0.03	0.03	0.00	3.01	1.14	0.55	0.30
LIVER	--	--	0.66	0.41	0.93	0.55	4.37	0.56	6.18	0.45
SPLEEN	--	--	0.91	0.61	0.09	0.06	1.21	0.40	0.12	0.04
RT. KIDNEY	--	--	0.33	0.14	0.07	0.03	18.59	3.43	3.80	0.45
MUSCLE	--	--	0.03	0.01	0.33	0.07	1.43	0.61	14.76	5.66
BONE	--	--	0.09	0.04	0.23	0.08	0.57	0.07	1.47	0.17
STOMACH	--	--	0.35	0.24	0.13	0.05	1.23	0.41	0.51	0.14
UPPER G.I.	--	--	0.10	0.03	0.11	0.02	1.08	0.13	1.32	0.32
LOWER G.I.	--	--	0.10	0.02	0.11	0.04	1.11	0.13	1.29	0.44

These issues can only be resolved by further experimentation. A dependable vesicle labelling agent is needed to determine whether the antibodies are staying with the vesicles.

CONCLUSION

This study showed that very good specificity can be obtained by coupling antibodies to vesicles. A significant fraction of the antibodies bound to vesicles were still viable, and showed good binding to the specific antigen on cells. The technetium labelling procedure proved reversible; the label would leave the vesicles and bind to cells when they were incubated together. A more reliable vesicle labelling procedure needs to be developed. The antibody did not show good tumor localization, but many questions remain as to the specific behavior of the antibody-vesicle complex in vivo. The further experimentation necessary along these lines is beyond the scope of this thesis.

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ACKNOWLEDGEMENTS

Throughout my time at M.I.T., there have been a number a very special people with whom I have had the pleasure of associating. Alan Davison has been an inspirational research supervisor, always believing in my abilities even when I was not sure of them myself. Alun Jones was equally supportive, and was always helpful during my otherwise solo journey into the fields of protein modification, immunology, cell fusion, cloning, cell culture, tumor growth, surgery, and dissection. Alan, Alun, and I almost never knew the answers to each other's questions, but somehow we managed to get beyond the problems.

The members of my research group have been without peer. Chris "Big Youth" Orvig taught me much about how to survive graduate school. He did his best to convince me of the virtues of Boston, while I was always extolling the virtues of California. After a year of these discussions, I decided Boston was not such a bad place and Chris moved to California. I am not sure if that means anything.

Mike "His Oneness" Abrams showed me that Zen and bluegrass were not mutually exclusive, you just have to approach life from a little different angle. His enthusiasm and talent were infectious, and he was responsible for laying the groundwork for this thesis.

In spite of our diametrically opposed views, James "Big Balls" Brodack and I managed to remain friends through a process that I still do not completely understand.

David "The Weasel" Brenner and I share a common interest in the monetary aspects of civilization. I hope David will be as fulfilled in his new direction as he has been disappointed in Chemistry.

The other members of the group (Bruno DePamphilis, Jim Kronauge, Karen Linder, John Lister-James, Rick Simon(?) and Alan Toothaker) have each been unique and a pleasure to be associated with. Even though considered "too rude and crude" by some, we are a good group.

I greatly appreciate the patience and kindness of JoAnn Sorrento, especially during her typing of this thesis. None of us could have made it without somebody keeping things afloat, and JoAnn was wonderful in her ability to take care of us.

The people at New England Nuclear Corp. (Brian Gallagher, Howard Sands, Bill Neacy, Peter Jones, Rich Harrison, Sue Danskin, and Beverly Brown) all were very helpful at various stages of this work. Teaching a chemist to do "biology" was probably not easy for them.

Last, but certainly not least, I would like to thank my wife Rona, my parents Robert and Cherry Carroll, and my brother Ken, who both collectively and individually have helped me make it to where I am. I appreciate the sometimes great effort and hope I will someday be able to return the favor.

BIOGRAPHICAL NOTE

The author was born January 2, 1960 in Glendale, California. He graduated from Brea-Olinda High School (Brea, California) in 1976 and received the B.S. Degree in Chemistry, with distinction, from Harvey Mudd College in 1980. After being railroaded into attending M.I.T., he began his doctoral research under Professor Alan Davison. After graduation this Spring, the author plans to continue in the field of nuclear medicine either as a postdoctoral fellow or research scientist.