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REVIEWS

Radiohalogenation of Proteins: An Overview of Radionuclides, Labeling Methods, and Reagents for Conjugate Labeling

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I. INTRODUCTION

Methods of radiolabeling proteins have been of interest for a variety of applications for several decades. Although many different radionuclides have been used to radiolabel proteins (1-7), the largest number of labeled-protein studies have used radionuclides of iodine, principally iodine-125 and iodine-131. These radionuclides of iodine have properties which are adequate for a number of different applications, and the radionuclides are relatively

easy to use and readily available at a nominal cost from commercial sources. Other radionuclides of the halogen group, while not studied extensively to date, could potentially be utilized in protein labeling. In fact, there are a number of radiohalogen nuclides, with a wide range of half-lives and radiochemical properties, which could be used for a variety of different purposes. As a group, radiohalogens may be particularly useful for radiolabeling of proteins because (a) their chemistry is well-understood (perhaps with the exception of astatine), (b) they form stable covalent bonds, (c) their steric and electronic nature can be expected to cause minimal alteration to the protein, (d) high specific activity radiolabeling can be accomplished, and (e) radionuclides with many different half-lives and photon or particle emissions are obtainable.

The first radiohalogen labeled protein studies may be that of radiobrominated human serum albumin (HSA) reported in 1943 by Fine and Seligman (8). Iodination of serum albumin was reported as early as 1945 (9); however, it appears that the first radioiodinated protein, iodine-131-labeled rabbit antiglobulin, was reported in 1948 by Pressman and Keighley (10). Radioiodination of proteins was quickly adopted in many studies and in 1957 Bale and Spar (11) wrote that "coupling of a radioactive halogen, almost always I^{131} , to an antibody or antigen has been the most widely used method of labeling with radioactivity for immunological purposes". They further stated that "coupling reactions are relatively easy to carry out in a routine fashion". In those early studies the radiohalogen label was used because prior studies involving labeling of the protein via incorporation of sulfur-35 ($t_{1/2} = 87$ d, β^-), tritium ($t_{1/2} = 12.35$ y, β^-) or carbon-14 ($t_{1/2} = 5730$ y, β^-) labeled amino acids resulted in very low efficiency of isotopic labeling. In a broader perspective, the advantages of using photon-emitting radionuclides rather than weak β -particle emitters such as tritium or carbon-14 for radiolabeling proteins went further than radiochemical yield. Importantly, counting biological samples labeled with photon (e.g. X-rays or γ -rays) emitting radionuclides can be done more easily than counting samples labeled with β -emitting radionuclides due to the need for tissue homogenization and mixing with scintillation cocktails for liquid scintillation counting. Perhaps more important though, many applications of radiolabeled proteins require that high specific activities be obtained for detection at very low levels of protein. As a comparison, the specific activity obtainable for iodine-125 (17 Ci/mg, 2125 Ci/mmol) or iodine-131 (10–50 Ci/mg, 1310–6550 Ci/mmol) labeled proteins allows for a much more sensitive assay of radioactivity, which has been estimated to be 75–150 times higher for radioiodine than for tritiated proteins and 35 000 times higher than for carbon-14-labeled proteins (12, 13). Indeed, it was the high specific activity obtainable with radioiodinated proteins which made the development of radioimmunoassay systems possible (14, 15).

The most common procedure employed in the radiohalogenation of proteins or peptides has been, and will likely continue to be, the reaction of an in situ prepared electrophilic radioiodine species with functional groups on a native protein, often referred to as "direct" labeling. The chemistry of direct labeling with radioiodine has been extensively studied and a number of reviews covering that chemistry have appeared in the literature (2, 3, 5, 16–18). Unfortunately, while direct labeling works very well for radioiodine, it is generally of little or no value for radiohalogenations with other elements in the halogen group. For example, direct labeling with bromine radionuclides requires harsh oxidizing conditions which can

cause denaturation of proteins, except when enzymes are used as the oxidants (19–21). However, routine use of enzymes for the radiobrominations is not practical at this time due to the cost and availability of the enzymes. Additionally, it might be expected that the harsh oxidizing conditions required to prepare electrophilic chlorine and fluorine would exclude use of radionuclides of these elements from direct radiohalogenations, and no reports of direct labeling radionuclides of these elements were found. Direct labeling of proteins with astatine nuclides can also be accomplished (22–25), but the astatine–protein bond produced has been found to be unstable (26, 27).

An alternative to direct radiohalogen labeling of proteins is conjugation of a small radiohalogenated molecule to the protein. Radiohalogenations using small molecule conjugates are more difficult to conduct because they involve more chemical steps, and often result in lower radiochemical yields, than direct labeling. However, conjugate labeling offers some benefits which cannot be obtained by direct labeling. Most important, perhaps, is the fact that conjugate labeling provides a method of introducing radiohalogens into proteins that cannot be directly labeled. Other benefits of conjugate labeling include providing (a) a method of stabilizing radiohalogens to in vivo dehalogenation by enzymes, (b) a method of labeling that does not expose the protein to harsh oxidants and reductants, (c) a method of labeling which permits dual-labeled-protein studies using the same molecule to attach two different halogen radionuclides, and (d) a method of labeling which can potentially provide some control of the secondary distribution of the radiohalogen.

This review focuses on protein labeling by conjugation of radiohalogenated small molecules. It is expected that peptides of less than 50 amino acids might be radiohalogenated in the same, or similar, manner as the larger proteins; however, they are not specifically addressed in the review. The chemistry of radiohalogenated conjugates in the review has been divided into three areas which are presented separately. The areas include (1) properties and chemistry of radiohalogens, (2) chemistry of radiohalogen labeling of small molecules, and (3) chemistry associated with conjugates for labeling proteins. A listing of radiohalogenated compounds that have been coupled (conjugated) to proteins is provided in Tables IV–IX. Structures for the compounds are provided in the tables and are referenced by compound number in the text. Some of the examples of conjugates cited are taken from preliminary communications (i.e. abstracts), because it was thought that this information should be included for completeness. In some literature citations, yields for the radiohalogenations or conjugations were not given directly, so where possible an estimation was made based on the information provided. When this was not possible, the notation of NR (not reported) was made.

Review of the literature has shown that there have been a large number of radiohalogenated protein conjugates prepared, with many new conjugates being reported within the past five years. Although an attempt was made to provide a complete list of halogenated conjugates studied to date, it is anticipated that some conjugates may have been overlooked as they have been reported in such diverse literature.

II. RADIONUCLIDES OF HALOGENS

A number of radionuclides, each with a different half-life and particle-emission properties, can be produced in the halogen group of elements. While iodine-125 and iodine-131 have been the radionuclides primarily used in

Table I. Selected Halogen Nuclides

nuclide	half-life	major route of decay ^a	ref
Iodine Nuclides			
¹¹⁹ I	19 min	β^+	29
¹²⁰ I	1.35 h	β^+ (81%)	32
¹²¹ I	2 h	β^+	29, 33
¹²² I	3.6 min	β^+ (76%)	30-32
¹²³ I	13.2 h	EC (100%)	28, 31, 32
¹²⁴ I	4.2 d	EC (70%), β^+ (30%)	29, 32, 45
¹²⁵ I	60.0 d	EC	29, 32
¹²⁶ I	13 d	EC (55%), β^- (44%), β^+ (1%)	29
¹²⁷ I	stable (100%)	none	29
¹²⁸ I	25 min	β^- (94%), EC (6%)	29, 32
¹²⁹ I	1.7×10^7 y	β^-	29
¹³⁰ I	12.5 h	β^-	29
¹³¹ I	8.04 d	β^-	29, 32
¹³² I	2.30 h	β^-	29, 32
¹³³ I	21 h	β^-	29, 32
¹³⁴ I	52.5 h	β^-	29
¹³⁵ I	6.7 h	β^-	29
Bromine Nuclides			
⁷⁴ Br	40 min	β^+ , EC	28, 29, 33
⁷⁶ Br	97 min	β^+ (75.5%), EC (24.5%)	28, 31, 32
⁷⁸ Br	16 h	β^+ (57%)	28, 32
⁷⁷ Br	57.1 h	EC (99.3%), β^+ (0.7%)	28
^{78m} Br	6.4 min	IT	29
⁷⁸ Br	<6 min	β^+	29
⁷⁹ Br	stable (50.5%)	none	29
^{80m} Br	4.5 h	IT	29, 31
⁸⁰ Br	18 min	β^- (92%), β^+ (3%), EC (5%)	29
⁸¹ Br	stable (49.5%)	none	29
⁸² Br	35.3 h	β^-	29, 32
⁸³ Br	2.4 h	β^-	29
⁸⁴ Br	32 min	β^-	29
Chlorine Nuclides			
^{34m} Cl	32.4 min	β^+ (53%), IT (47%)	29, 32
³⁵ Cl	stable (75.5%)	none	29
³⁶ Cl	3×10^5 y	β^- , β^+	29, 32
³⁷ Cl	stable (24.5%)	none	29
³⁸ Cl	37.2 min	β^-	29-32
³⁹ Cl	1 h	β^-	29
Fluorine Nuclides			
¹⁸ F	109.8 min	β^+ (97%), EC (3%)	28, 31, 32, 36
¹⁹ F	stable (100%)	none	
Astatine Nuclides			
²¹¹ At	7.21 h	α (42%), EC (58%)	28, 31, 32, 35

^a β^+ = positron emission, β^- = negatron (electron) emission, EC = electron capture, IT = isomeric transition.

protein labeling, there are a number of different halogen radionuclides that could be used for unique or specific applications. The choice of radionuclide to use for a particular application will be dependent on the nuclear emission properties, the physical half-life, the decay characteristics, the daughter nuclide characteristics, and the cost and availability. While many of the radiohalogen nuclides are not commercially available, most are obtainable through activation of the appropriate target materials using reactor (neutron) or cyclotron (proton, deuteron, or alpha) irradiations. However, it must be emphasized that even though a radionuclide can be produced, issues such as availability of target material, radioisotopic purity from the irradiation, quantity of radionuclide needed, and ultimately the cost may preclude its use in an application. Production of radionuclides of halogens is outside the scope of this review, but can be found in a number of articles from the literature (28-36).

The half-lives and primary emissions of halogen radionuclides which may have an application to protein labeling are given in Table I. Radionuclides with half-lives shorter than 3 min are not included, as these are unlikely to be of value for protein labeling. Since the ultimate use of the radiohalogen-labeled proteins will

define which of the radionuclides are of interest, the following discussion is structured according to application. The presentation is not a review of the area, but rather an overview with basic information to acquaint the reader with some of the important aspects of the area. There will be many potential applications not mentioned herein, and many more that have not yet been envisioned.

A. Halogen Radionuclides for in Vitro Applications. Iodine-125 has been the primary radionuclide for in vitro applications due to its 60-d half-life and low-energy photon emissions. The long half-life permits the preparation and storage of labeled protein for extended periods prior to usage. The lack of particulate emissions and low-energy photon emission makes it particularly attractive for storage, as minimal radiation damage to the labeled protein is expected. Specific activities that are very near the theoretical value (17.2 Ci/mg, 2175 Ci/mmol) are obtained with iodine-125. Its cost and availability are also very reasonable. In review other halogen radionuclides, it appears that there are no other radionuclides that can readily be used in the place of iodine-125 for in vitro studies.

B. Halogen Radionuclides for in Vitro Imaging. Any of the radionuclides of halogens which have X-ray or γ -ray (photon) emissions might be considered for in vivo imaging applications, but the extent of usefulness of the radionuclide to following the distribution and pharmacokinetics of a radiolabeled protein in vivo will be dependent on a number of factors. Most important are the energy and abundance of the photon emission(s) and the biological system studied. The application of radionuclides for measurement of receptor-ligand binding in vivo (37) has become less restricted through the advent of new imaging equipment and related computer image enhancement employed in single photon emission tomography (SPECT) and positron emission tomography (PET). Advancements are rapidly changing the detection limitations of specific radionuclides as outlined in the scientific highlights of the annual meeting of The Society of Nuclear Medicine presented by Dr. Wagner (38-40).

Evaluations of labeled proteins in vivo are very important applications of radiolabeled proteins. While analysis of materials in excrement (e.g. urine or fecal material) permits gaining information on the clearance rate of labeled protein and of some of the pathways in the metabolism of the labeled protein, it provides little knowledge of the in vivo distribution of protein. In vivo data can be obtained by sacrificing animals, excising tissue samples, and counting the radioactivity in an appropriate counter. Tritium- and carbon-14-labeled proteins can be used to obtain in vivo data, but due to the relative ease of counting tissue samples, the photo-emitting radionuclides iodine-125 and iodine-131 are routinely used. From experiments using iodine-125 and iodine-131, biodistribution data, pharmacokinetic data, and data on the metabolic fate of the radiolabel can readily be obtained. However, iodine-125 and iodine-131 are not optimal to assess the parameters in patients. Iodine-125, while it can be imaged by a thin-crystal γ -camera (marginally) when evaluating small animals such as mice, it is of little or no value in imaging patients due to the attenuation of the photons through tissues. Iodine-131 is often used for imaging with conventional γ -cameras, but its high-energy γ -emissions are not optimally counted, and its β -particle emission results in more radiation dose to the tissues than is desirable. An alternate radionuclide of iodine, iodine-123, has a reasonable half-life of 13 h and a nearly ideal γ -emission of 159 keV, in high abundance (100%) for imaging with the current γ -cameras. Unfortunately, production of this

radionuclide without radionuclidic impurities is expensive, which makes its general use impractical. Much controversy has accompanied this radionuclide as it has been felt that its use would be greatly expanded if it could be obtained inexpensively, while others have felt that it could be made inexpensively if its use was more in demand (41). Another γ -emitting radiohalogen that has been considered for *in vivo* diagnostics is bromine-77. While its emitted photons at 239 keV (24%) and 521 keV (23%) are high for efficient detection with current γ -cameras, bromine-77 has been used in imaging (42). Unfortunately, while it can be produced via a number of routes (28), routine production of curie quantities of bromine-77 by high-energy spallation reactions (34), which was carried out at Los Alamos National Laboratory, has stopped.

Perhaps the area that holds the greatest promise for new applications of radiohalogens is PET imaging in medicine (43). PET studies can provide quantitative data on the distribution and pharmacokinetics of radiohalogenated proteins *in vivo* (44). There are a number of positron-emitting radionuclides of halogens, with a variety of physical half-lives and emitted-positron energies. The positron energy (E_{max}) is very important, as this defines the maximum distance that the positron will travel before its annihilation will occur, which in turn sets some limitations on the best resolution that can be obtained from the imaging device (i.e. tomograph). For positrons which have emission energies of 2 MeV or above, this distance may be in centimeters and can cause severe limitations in the resolution. However, radiohalogen nuclides with high-energy positrons may be useful in obtaining pharmacokinetic data.

The most widely used positron-emitting radiohalogen is fluorine-18. Fluorine-18 is likely to be the nuclide of choice in many protein-labeling applications as it (1) can be readily produced via a number of different methods (28, 36), (2) has a high positron abundance (97%), and (3) has a relatively low positron emission energy (0.635 MeV max), which limits its range to 2.3 mm in water (or tissue). However, fluorine-18's 110-min half-life may limit its use for some applications with proteins. Chlorine-34m is also a positron emitter, but it has a shorter half-life, lower positron-emission rate, and higher positron energy than fluorine-18 (28). Bromine-74m is another positron-emitting halogen radionuclide. It has a shorter half-life and a higher positron energy than fluorine-18 (28). Another bromine radionuclide, bromine-75 has a half-life of 97 min, which is similar to that of fluorine-18, but it has a positron emission of 1.74 MeV in 75% abundance. While bromine-75 may be produced at higher specific activities than fluorine-18, unless there are significant difficulties in labeling of proteins with fluorine-18, it is not likely to be used in its place. Yet another positron-emitting radionuclide or bromine, bromine-76, has a half-life of 16 h and appears to be an attractive candidate for labeling of proteins that have longer biological half-lives (e.g. 1–3 d). A positron-emitting radionuclide of iodine, iodine-122, will probably not be of general use for protein labeling due to its very short half-life (3.6 min). However, the fact that iodine-122 can be produced from a generator system makes it attractive when applicable (31). A longer lived positron-emitting radionuclide of iodine, iodine-124, has been investigated as a radiolabel. It has a 4.2 d half-life, but until recently it was not considered a reasonable candidate due to its complicated decay emission properties. However, it has been studied as a label for intact monoclonal antibodies since its half-life matches the biological half-life of antibody (45).

C. Halogen Radionuclides for Therapy. Radiohalogens with particulate emissions are being tested or considered for *in vivo* therapy of cancer, particularly in combination with monoclonal antibodies (46–49). Iodine-131 has been the most widely used therapeutic radionuclide for labeling antibodies. Due to its availability and cost, it is likely to continue to be used for this purpose, although iodine-131 does not have optimal characteristics for therapy (50) and its high energy (i.e. 364 keV) γ -emissions pose a radiation exposure risk to the people working with patients undergoing treatment. Thus, it is likely that other radionuclides may be used in the future (50–53). Of the β -particle-emitting radiohalogens, bromine-82 appears to be an alternative to iodine-131. A major difficulty with the use of bromine-82 is that it is generally produced from neutron irradiation of bromide salts (e.g. NH_4Br), which results in too low a specific activity for protein labeling. Further, the difficulties of working with patients treated with hundreds of millicuries of bromine-82 would be expected to be worse than that with iodine-131 as it has a number of high-energy γ -emissions (e.g. 554, 619, 776 keV). Other β -particle-emitting radiohalogens with shorter half-lives may be useful as well.

Potentially, the positron-emitting radiohalogens fluorine-18, bromine-75, bromine-76, and iodine-124 could be used for therapy, with the added benefit of being able to assess patient radiation dosimetry to tissues and monitor changes in tumor volume through PET scans. However, the difficulties mentioned regarding radiation risk to patient care providers would also apply since positron annihilation results in the emission of a 511 keV photon. Further, difficulties in producing some of the nuclides may limit their general use.

Another halogen radionuclide that may hold potential for some therapy applications is astatine-211 (54, 55). While its half-life of 7.2 h is relatively short, it decays by α -particle emission, which is extremely cytotoxic, such that it could be an effective therapeutic agent if it were specifically delivered to the cell or target material (53). Basic science studies are needed to understand the limitations of using proteins labeled with α -particle-emitting radionuclides for therapy. At present many investigators believe that astatinated antibodies may be limited to therapy of metastatic or blood-borne disease due to the short path length of the α -particle. Investigations on the therapeutic usefulness of astatine-211 have been very limited due to the difficulty in obtaining the radionuclide.

Auger electron emitters have been shown to be effective at cell killing if they can be incorporated into nuclear materials in cells (56). Iodine-125 decays with an emission of a number of Auger electrons; however, its 60-d half-life may be problematic if the turnover of labeled protein in the nucleus is considerably shorter than that. An antibody labeled with iodine-125 reactive against the α -epidermal growth factor receptor, which is internalized (57), in human glioma cells has been shown to be cytotoxic (58). Bromine-77 which decays by electron capture has also been tested for the cytotoxicity of the emitted Auger electrons, and found to be effective at cell killing when incorporated into nuclear material as [^{77}Br]bromodeoxyuridine (56). This radionuclide was thought to have the advantage that it had a shorter half-life than iodine-125 and that bromine was not taken up by the thyroid. Another iodine radionuclide, iodine-123, also has emission of Auger electrons. Due to its shorter half-life, and a γ -emission that is easily imaged, this radionuclide is an attractive candidate to study for therapeutic applications.

D. Other Halogen Radionuclides. Protein labeling with iodine-132 was reported in 1955 (59). Iodine-132 was obtained from tellurium-132, a fission product. It is interesting that this radionuclide, which has a much shorter half-life (2.3 h) than iodine-131, has not gained more prominence. However, the availability and cost of iodine-131 appears to have alleviated a need for it. More recently, the very long half-lived iodine-129 (1.57×10^7 y) has been used as an *in vivo* tracer for protein turnover (60). The plasma concentration of iodine-129 can be assessed accurately using by neutron-activation analysis. Unfortunately, while a patient may receive less of a radiation dose with this radionuclide, its use must be tempered by the need to dispose of such a long half-life radionuclide in the environment.

III. RADIOHALOGEN LABELING

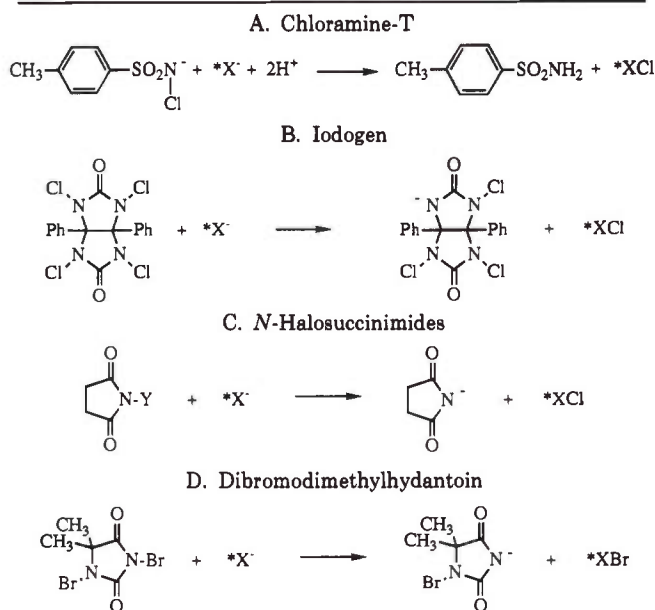
Methods for radiohalogenation of proteins, in general, should be rapid and give high radiochemical yields. The radiohalogenated protein that is obtained should be of high specific activity and should be labeled in a manner that results in a stable attachment of the radiohalogen. While the chemistry associated with radiolabeling using radiohalogens is the same as that of halogenation with stable nuclides, there are some distinct differences in reactions due to the fact that radiohalogenations are generally very dilute and contain many minor impurities from processing of the target material and in the chemicals used which are present in more mass than the radiohalogen itself. Thus, optimization of reaction parameters is almost always a requisite of radiohalogenations.

Stability of the radiohalogen-labeled protein is very important if one wants to be assured that the observation of radioactivity is a true indicator of what is happening with the protein. Most radiohalogenations, with the exception of fluorinations, are conducted as electrophilic substitution reactions (61). The rate of electrophilic substitution reactions is a distinct advantage with the dilute radiohalogens, allowing the reactions to occur rapidly under mild conditions. Electrophilic reactions require the production of an electrophilic radiohalogenation reagent through oxidation of halide ions. A section on oxidants used in electrophilic radiohalogenations is given below. Electrophilic radiohalogenation reactions are terminated prior to workup, and a section on this reaction step is also included below. Other parameters of radiohalogenations, such as solvent selection and purification method, are included in the discussion of these two topics and are not addressed separately.

Radiohalogenation reactions of proteins and small-molecule conjugates is presented below by the nature of the molecule being halogenated. A large amount of research has been conducted on high specific activity radiohalogenations of compounds for radiopharmaceutical development over the past 2 decades (62, 63). Many of the methods developed for high specific activity radiohalogenations can be, and in some examples have been, directly applied to radiohalogenation of proteins and small-molecule conjugates. Indeed, a number of the radiohalogenated small molecules used to prepare radiopharmaceuticals could potentially be used as protein conjugates.

A. Halogen-Oxidizing Reagents. Radiohalogens are generally obtained as their halide salts and must be oxidized to form an electrophilic halogenating species to label molecules. Oxidation of chloride, bromide, iodide, and astatide can readily be accomplished by inorganic reagents such as MnO_4^- , Ce^{4+} ion, $\text{Cr}_2\text{O}_7^{2-}$, and IO_3^- . However, the use of such strong oxidants is incompatible

Table II. *N*-Haloamine Oxidizing Reagents^a



^a Where X = F, Br, I, or At; Y = Cl (*N*-chlorosuccinimide), Br (*N*-bromosuccinimide), I (*N*-iodosuccinimide).

with most organic molecules and requires that the halogen be distilled from one vessel to another. Many of the early radiohalogenations of bromine used these oxidants (64–69), but the preferred approach for all of the radiohalogens today is to generate the electrophilic radiohalogenation reagent *in situ*. *In situ* oxidation can be accomplished by using dihalogens (X_2) of the same element, but in most examples this is not desired as the halogen being employed will react with the compound of interest and this will result in a decrease in the specific activity obtained. Further, oxidation to form a dihalogen species (X_2) results in a maximum radiochemical yield of 50%. The maximum radiochemical yield can be increased to 100% when a mixed halogen species, such as halogen chloride (e.g. ICl), is generated.

There are very small quantities of radiohalide present when high specific activity solutions are employed, with the exception of radiohalogenations using therapeutic quantities of radiohalide. Even though microgram quantities of oxidant are used (often in the 10–100 $\mu\text{g}/\text{mCi}$ range), the stoichiometry of oxidizing reagent *in situ* is many times greater than that of the halide. This fact makes it necessary to have oxidants that do not adversely affect the molecules being labeled. For direct labeling of proteins (with radioiodine), the oxidant should be compatible with aqueous solutions of proteins, should not denature the protein, and should permit easy separation when done. Various oxidizing reagents have been used for *in situ* generation of mixed halogens. Iodine monochloride has been used as a method of iodination of proteins (70). The most common oxidizing reagents used are the *N*-haloamine compounds chloramine-T and iodogen shown in Table II (71, 72). These compounds are commercially available and the literature has many examples of their use. Chloramine-T, sodium *N*-chlorotoluenesulfonamide, has been used extensively for oxidation of halides and its chemistry is well-known. This reagent is water-soluble and has been utilized for *in situ* oxidation of iodide, bromide, and astatine. A similar reagent, iodogen, 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril, has also been used extensively in radioiodinations. Iodogen (IDO-GEN) is very stable and is virtually insoluble in water. This latter

fact has made iodogen attractive because it is readily separated from the radiolabeled protein. Other related oxidizing reagents include IODO-BEADS, where chloramine-T is attached to a solid support (73).

Radiohalogenation of small molecules to be used as protein conjugates has different requirements of oxidizing reagents. Since the reactions are conducted in a separate vessel from the protein, stronger oxidizing reagents can be used without affecting the protein. Many examples of chloramine-T oxidations of halides for radiohalogenations of small molecules have been reported (74–76). The reactions have generally been conducted in aqueous solutions, but the salt is also soluble in some aprotic solvents such as trifluoroacetic anhydride (77). The use of various oxidizing reagents in high specific activity radiohalogenations has been reviewed by Coenen, Moerlein, and Stocklin (78). In preparing radiolabeled small molecules for protein conjugation, Zalutsky and co-workers have used *tert*-butyl hydroperoxide (TBHP) in chloroform/acetic acid solution to oxidize radioiodide and astatide (79, 80). In our protein conjugate studies, *N*-chlorosuccinimide (NCS) (see Table II) in methanol/acetic acid has been used for oxidation of radiobromide, radioiodide, and astatide (81–83). This reagent is readily available and presumably produces XCl when reacted with chloride, bromide, iodide, or astatide (84, 85). NCS has been attractive because it is a weaker oxidant than *tert*-butyl hypochlorite (86), *N*-chlorotetrafluorosuccinimide (87), or dichloramine-T (88). NCS does not halogenate phenolic compounds, and thus, does not dilute the specific activity of most radiohalogen-labeled compounds by producing chloro derivatives. A drawback to the use of NCS is the fact that only small quantities (<10%) of water can be present without adversely affecting the oxidation.

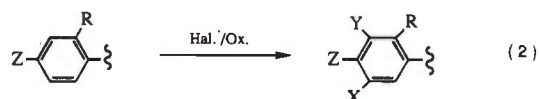
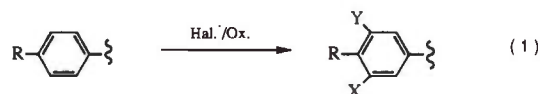
Another method of oxidizing radiohalogens in situ for labeling proteins or small molecules is to use oxidative enzymes. Radioiodinations and radiobrominations have been successfully conducted using enzymes (18). Lactoperoxidase, horseradish peroxidase, and myeloperoxidase, have been used for radioiodination of proteins. The enzymes chloroperoxidase (19), myeloperoxidase (20), and bromoperoxidase (21) have been successfully used in radiobrominations of monoclonal antibodies. While the use of enzymes in radiohalogenations offers a very mild method of oxidation, routine use of enzymes to radiohalogenate proteins is unlikely as (1) the enzymes can themselves be radiohalogenated, (2) the radioiodinated proteins have to be separated from the enzyme [although a solid support enzyme can be used (89)], and (3) the enzymes are more expensive and most are more difficult to obtain than the commonly used organic oxidants (i.e. chloramine-T, iodogen).

B. Radiohalogenation Reaction Termination. Electrophilic radiohalogenation reactions are routinely stopped (terminated) prior to workup due to concerns for volatile radiohalogens and subsequent undesired reactions. Depending upon the radionuclide, oxidant and solvent(s) used, and concentration of reagents (and perhaps the whim of the investigator), radiohalogenation reactions are allowed to proceed from 30 s to 30 min. Many radiohalogenation reactions are complete almost instantly. To terminate the reaction, an aqueous solution (or phosphate buffer solution) of the reductant sodium metabisulfite is generally added to reduce the electrophilic halogen species to the halide. A minimum amount of reducing agent should be added to quench (terminate) the reaction such that the possibility of side reactions is minimized. Investigators generally use quantities of sodium metabisulfite

ranging from 25 $\mu\text{g}/\text{mCi}$ (90) to 125 $\mu\text{g}/\text{mCi}$ (91) when terminating direct labeling reactions. In many direct radioiodination reactions of monoclonal antibodies, investigators have eliminated the reduction step and have simply put the radiolabeled protein over a size-exclusion column such as a Sephadex G-25 (PD-10) (92), Sephadex G-50 (93), Biogel P-60 (94), Pharmacia G-10 (95), or a Dowex column (96, 97). This process would be expected to leave the halogen on the column. One must be cautioned that this practice does require careful handling techniques and careful cleanup of reaction vial and column (see VI. Radiation Safety Considerations).

When a small molecule is radiolabeled prior to conjugation, it is necessary to quench unreacted halogen since any excess could react with the protein during the conjugation step. If one is concerned about deleterious effects of the metabisulfite on the protein, excess radiohalogen can be removed by an evaporation of the solvent under a gentle stream of N_2 or argon. The solvent evaporation must be done in an enclosed vessel which has an exit for the gas that passes through a charcoal bed. The volatilization of radiohalide should be avoided if at all possible, and other methods of terminating the reactions are preferred. Bolton has described the use of a cysteine solution for reaction termination (98). Another method of removal is to add a reactive species that can be readily separated from the labeled protein, such as phenol, to trap the electrophilic radiohalogen.

C. Radiohalogenation of Activated Aryl Compounds. 1. *Compounds Containing "Strongly Activating" Groups.* Aromatic ring compounds substituted with OH (phenols), NH_2 (anilines), or NHR or NR_2 (N-alkylanilines) are "strongly activated" toward electrophilic halogenation (99). These compounds react rapidly with electrophilic halogens, which is particularly attractive for reactions with the very dilute high specific activity radiohalogens. When the phenol hydroxyl ($\text{R} = \text{OH}$) or aniline amine ($\text{R} = \text{NH}_2$) is situated para to the aryl position containing the conjugation group, halogenation can lead to two products, as depicted in eq 1. These products are

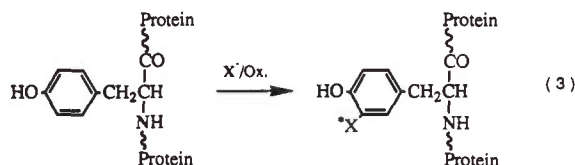


the monohalogenated product ($\text{X} = \text{halogen}$, $\text{Y} = \text{H}$) and the dihalogenated product ($\text{X} = \text{Y} = \text{halogen}$). When the activating substituent is in either the 2 or the 3 position of the aryl ring, three halogenated products can be obtained as depicted in eq 2. These products are either of the monosubstituted products ($\text{X} = \text{halogen}$, $\text{Y} = \text{H}$ or $\text{X} = \text{H}$, $\text{Y} = \text{halogen}$) or the disubstituted product ($\text{X} = \text{Y} = \text{halogen}$).

2. *Compounds Containing "Mildly Activating" Groups.* Aryl compounds containing OR (e.g. anisoles, where $\text{R} = \text{CH}_3$), NHCOR (e.g. acyl anilines), OCOR (e.g. phenolic esters), and SR (e.g. thiophenol ethers) can be considered "moderately activated" aromatic ring compounds in electrophilic aromatic substitution reactions (99). Depending on the nature of other substituents present, aromatic rings containing these functionalities can also be readily substituted with electrophilic halogen species. While mildly activated aryl compounds can be used for radiohalogenations, they are not as attractive as highly activated

aryl ring compounds as minor quantities of more reactive impurities could significantly lower radiochemical yields due to side reactions with the impurities. Additionally, the same type of mixtures of halogenated products can be obtained in the electrophilic aromatic substitution reactions as described for aryl compounds containing strongly activating groups.

3. Direct Labeling of Proteins. Several functional groups present in protein structures are reactive with electrophilic radioiodine. These include the highly activated phenolic ring of tyrosine residues, the imidazole ring of histidine residues, the benzene ring of phenylalanine residues, the indole ring of tryptophan residues, and the sulfhydryl groups of cysteine residues (100). The most readily iodinated residues are tyrosine, histidine, and cysteine. Under various reaction conditions (e.g. low pH) differing percentages of the tyrosyl, histidyl, and cysteinyl moieties have been observed to react with radioiodine (101). However, using commonly employed reaction conditions (i.e. pH 7.4, chloramine-T oxidant) a very large percentage of the radioiodine will be present on the phenolic tyrosine residues as depicted in eq 3 (102).



D. Radiohalogenation of Nonactivated Compounds.

Radioiodination of activated aromatic ring conjugates has been very useful; however, not all halogens can be introduced by the use of activated aromatic compounds. For example, astatine labeling of phenolic compounds is not practical, as the phenolic ring is too activated to produce stable compounds with astatine nuclides (103–105). Furthermore, halogenated phenols and anilines are susceptible to dehalogenation by nucleophiles, and radioiodinated tyrosine derivatives (and to a lesser extent other radioiodinated phenols) are susceptible to *in vivo* enzymatic dehalogenation (106–108). Because of their higher stability, nonactivated aromatic ring compounds and halovinyl-substituted compounds are good alternatives to activated aromatic rings for halogens.

Fluorine labeling is somewhat different from the other radiohalogenations as fluorine is not readily displaced from aliphatic carbons and can be introduced into aromatic ring compounds via ipso displacement reactions. Electrophilic fluorination of activated aromatic compounds has been difficult due to the high oxidizing characteristics of fluorinating reagents (99). A number of reviews on the chemistry of fluorination (109–112) and radiofluorination (113–116) are in the literature.

Electrophilic chlorination and bromination of nonactivated aromatic ring compounds can be achieved directly (117, 118), but a catalyst is often used (99). Electrophilic iodination of nonactivated or deactivated aromatic ring compounds often requires that harsh reaction conditions or catalysts be employed (119–124). High specific activity radiohalogenation of nonactivated compounds can be particularly difficult in these reactions, as there are many impurities present that could react with the halogen before it reacted with the aromatic ring. These factors led investigators to find methods of regiospecifically introducing radiohalogens into nonactivated aromatic rings and vinyl moieties. Two methods of halogenation have been found to be suitable to fulfill these needs. These methods are incorporation of radiohalogens via decomposition of

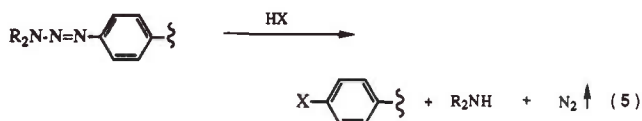
aryl diazonium salts and incorporation of radiohalogens in aromatic and vinyl positions in molecules using organometallic intermediates. Brief overviews of each of these methods of halogenation are given below.

1. Diazonium Salts and Triazines. One of the earliest methods developed for incorporation of halogens into nonactivated ring compounds was use of aryl diazonium salt decompositions [dediazoniations (125)] as depicted in eq 4. Reactions involving cuprous chloride and bromide



are commonly referred to as the Sandmeyer reaction (126, 127) and those involving HCl or HBr are referred to as the Gatterman reaction. There are a number of difficulties in using this method for radiohalogenations (78), with the most important being the low radiochemical yields that are generally obtained.

Introduction of astatine-211 via diazonium salt decomposition (128) gave higher radiochemical yields than those obtained for iodine-131 (129). Indeed, initial labeling reactions of organic compounds with astatine-211 were conducted with diazotized benzidine and *p*-aminobenzoic acid (130). Improvements in the stability of the aryl diazonium compounds and reaction conditions (e.g. no strong oxidants present) for halogenations and radiohalogenations were obtained by preparing triazines (131–134) as depicted in eq 5. This reaction is often referred to as the Wallach reaction.



Fluorination and radiofluorination have been accomplished by decomposition of the tetrafluoroborate salts of arene diazonium compounds, referred to as the Balz-Schiemann reaction (116) or Schiemann reaction (62). This reaction results in low specific activity labelings and is complicated by side products of the diazonium salt decomposition (135, 136).

2. Organometallic Intermediates. Radiohalogenation reactions using organometallic intermediates have been studied extensively. Halogen-reactive organometallic compounds have been studied as a means of efficiently, rapidly, and site-specifically incorporating radiohalogens into small molecules. Various research groups have investigated the use of organothallium compounds (137), organomercury compounds (138–141), organoboranes (142), organostannanes (143–147), organosilanes (148–154), organosilicates (155, 160), and organogermanes (157, 158). A generalized scheme for this reaction is given in eq 6. Small-molecule radiolabeling has been reviewed (78, 159) and will be only briefly discussed below.



There have been numerous studies by investigators involving radiohalogenations of aromatic compounds via organometallic intermediates. Based on the results obtained in studies of a particular organometallic intermediate, it is difficult to choose which one to use in designing

small molecules for protein conjugation. Some studies which have compared radiohalogenations of different organometallic intermediates have determined that for general applications organostannanes are probably the best candidates. For example, a comparative study of the application of arenes metalated with tin, mercury, germanium, boron, and silicon for introducing the very short half-life (3.6 min) iodine-122 into aromatic compounds has been reported by Moerlein, Mathis, and Yano (160). These investigators were interested in very fast (<5 min) and efficient reactions. They investigated a number of reaction parameters and concluded that high specific activity radioiodinations could be obtained in high yield (>90%) using aryltin or -mercury compounds, whether they were activated or deactivated toward electrophilic substitution. The investigators indicated that they felt that the arylstannane was the best candidate for labeling since the organomercury compounds were more difficult to obtain as a single regioisomer.

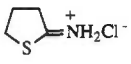
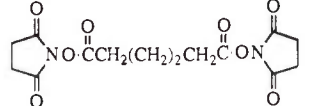
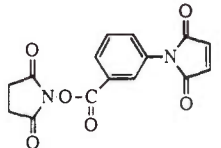
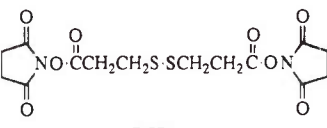
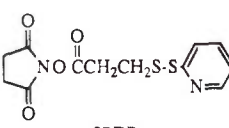
Our research group was also interested in comparing radioiodinations of organometallic compounds for incorporation of radioiodine into molecules that were being developed for protein labeling. Initial studies to assess which organometallic intermediate would be best for radiohalogenations were carried out in a comparison of low specific activity radioiodinations of 4-(methoxycarbonyl)mercurichalides, 4-(methoxycarbonyl)boronic acid, and 4-(tri-*n*-butylstannyl)benzoate (161). Later, no-carrier-added radioiodinations and astatine labeling of these compounds, along with 4-(tri-*n*-butylstannyl)toluene and potassium 4-tolylpentafluorosilicate, were also carried out (162). On the basis of the results of those investigations, arylstannanes were chosen as the organometallic intermediate of choice for radiohalogen labeling. This choice was made, even though the phenylboronic acid gave very good radiohalogen labeling yields at the no-carrier-added level, because the boronic acid was more difficult to synthesize and purify. Additionally, purification of the radiohalogenated small molecule from aryltri-*n*-butylstannanes is not necessary in some labeled protein applications due to a partitioning of the highly lipophilic arylstannane from the aqueous medium.

Vinyl halides are also stabilized toward nucleophilic displacement and have therefore been used as labeling groups to introduce radiohalogens into small molecules. Introduction of halogens into vinyl positions is facilitated by the use of organometallic intermediates. Some of the organometallic intermediates give addition products when reacted with electrophilic halogens (163); however, vinyl boronic acids and vinyl stannanes are cleaved to yield the vinyl halide directly as depicted in eq 7. These reactions have been studied as a method of producing radiohalogenated compounds (164). Organoboronic acids and stannanes have been used extensively for preparing radiohalogenated steroids (165–168). In another reaction, halofluorination of alkenes via BrF or IF produced in situ by reaction of fluoride with *N*-haloamines (NBS, NIS or 1,3-dibromo-5,5-dimethylhydantoin; Table II) yields a mixture of isomers which could be dehydrohalogenated to give vinyl fluorides (169).

IV. RADIOHALOGENATED CONJUGATES

There are many reviews that describe the chemistry of protein modification through conjugation of small molecules (170–173). The principal functional groups on proteins which are used in conjugation reactions with other molecules are amines, sulfhydryls, and oxidized sugars; however, other reactive groups such as phenols, carbox-

Table III. Protein Modification Reagents^a

A. Sulfhydryl-Generating Reagents		
	HS-CH ₂ -CH ₂ -OH	HS-CH ₂ -CH(OH)-CH(OH)-CH ₂ -SH
2-Iminothioline	β-ME	DTE or DTT
B. Protein Cross-Linking Reagents		
		
DSS		MBS
		
DSP		SPDP

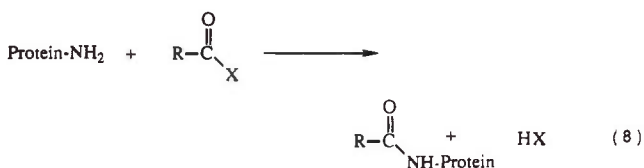
^a Where β-ME = 2-mercaptoethanol; DTT = dithiothreitol; DTE = dithioerythritol; DSS = disuccinimidyl suberate; MBS = *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester; DSP = dithiobis(succinimidyl propionate); SPDP = *N*-succinimidyl 3-(2-pyridylthio)propionate

ylates, and imidazoles may also be used if desired. Additionally, a number of reagents have been developed which can be used to produce functional groups (e.g. sulfhydryls) or reactive entities (e.g. maleimides) on proteins for conjugation reactions with other molecules. Examples of these latter reagents are shown in Table III and examples of their use are described in the text.

One of the primary concerns in the design of new conjugates for radiohalogenation of proteins is which functional group will be utilized in the conjugation reactions. Thus, the following text is subdivided into sections on the basis of which protein functional group reactions are to be carried out with the small-molecule radiohalogenation reagents.

A. Amine-Reactive Conjugates. The most common functional group used for conjugation of radiolabeled molecules to proteins is the amine group, generally ε-amino groups of lysine residues. Many compounds reactive with lysines have been studied (176). Compounds that contain amine reactive functionalities such as active esters, imidates esters, aldehydes, and isothiocyanate groups have been used in protein conjugations. A large number of reaction conditions for conjugations with proteins have been studied. The primary considerations in the conjugation reactions are pH (usually >8.0) and concentrations of protein and reagents. A list of amine-reactive radiohalogenation reagents is given in Table IV. A brief description of the chemistry associated with radiohalogenated amine-reactive compounds is given in the following text.

1. Active Esters. Coupling of amines with "active" esters provides small-molecule conjugations which have amide linkages to the protein as shown in eq 8. There are



a large number of different active esters that may be synthesized. Fortunately, the choice of which active ester compound to prepare can be made more readily by reviewing the literature on the use of active esters in peptide synthesis (177, 178). For conjugates of radiohalogenated compounds the most commonly used active ester is the *N*-hydroxysuccinimide ester.

Perhaps the first activated carboxylic acid compound used for radiohalogenated-small-molecule conjugation of a protein was reported by Blau, Johnson, and Pressman (179). In their study, dual-labeled *p*-[¹³¹I]iodobenzoic [14C]acid, **2a**, was prepared by lithiation/carbonylation of diiodobenzene to incorporate a [14C]carboxylate into *p*-iodobenzoic acid, **1**, followed by ¹³¹I exchange at 250–260 °C for 10–12 h in a sealed ampule contained in a pressure bomb. In this early study the benzoic acid chloride, **2b**, was prepared for conjugation to the a rabbit γ -globulin. Although the labeling time was very long and the specific activity of the labeled product (estimated to be in the nCi/mg range based on Table I values for benzoate) was not as high as what would be desired (e.g. mCi/mg range), this very early dual-labeling experiment demonstrated that a radioiodinated protein conjugate could be prepared which was stable in vitro and in vivo.

The need to radiolabel proteins with high specific activity radioiodine led initially to protein conjugates which contained phenolic rings, as these were readily radioiodinated with high specific activity radioiodine. Some 16 y after the reported protein labeling of Blau et al., Bolton and Hunter reported a new method of labeling proteins which resulted in obtaining high specific activities of radioiodinated proteins (180). In the reported studies ¹²⁵I-labeled *N*-succinimidyl 3-(4-hydroxyphenyl)propionate, **4**, was prepared, producing a mixture of products (two radioactive spots by TLC analysis). The radiolabeled species were most likely the monoiodo derivative **4a** and the diiodo derivative **4b**. This mixture of labeled compounds was dried and reacted in a 3–4 mol ratio with a protein at pH 8.5. Three different protein hormones were radioiodinated, resulting in different (but high) specific activities for each protein. The authors found that the use of the new reagent (Bolton–Hunter reagent) was at least as good a method of radiolabeling as direct labeling, and in some examples was superior. They reported that the new labeling procedure generally gave specific radioactivities and biological activity of the labeled protein similar to those found with direct labeling with chloramine-T. However, it had a much broader application, as it could be used to label proteins which did not contain tyrosine residues, or which were sensitive to the oxidizing or reducing conditions of direct iodinations. Subsequent to the initial studies, the Bolton–Hunter reagent has been used for labeling many different proteins, and a description of large-scale radiolabeling procedure using the Bolton–Hunter reagent has been reported (181). Reviews of proteins labeled by the Bolton–Hunter reagent have appeared in the literature (182, 183). The Bolton–Hunter reagent has also been used to label cell surface (184) and platelet surface proteins (185). The Bolton–Hunter reagent is commercially available from several sources as a reagent for radioiodination, or in the monoradioiodinated (mixture or HPLC purified) and diradioiodinated (crude or HPLC purified) forms in specific activities of 2000–4000 Ci/mmol. A more water-soluble sulfohydroxysuccinimide ester is also commercially available. The sulfohydroxysuccinimide ester of the Bolton–Hunter reagent has been applied to labeling of cell surface proteins and is particularly attractive, as the charged sulfonate group

does not allow the radioiodinated reagent to cross the cell membrane (186).

The Bolton–Hunter reagent has also been used to obtain radiobrominated proteins. Attempts to obtain monobrominated ⁷⁷Br-labeled Bolton–Hunter reagent at Los Alamos National Laboratory resulted in mixtures of monobrominated (**4c**), dibrominated (**4d**), and nonbrominated (**3**) reagent using conventional oxidants (187). The fully dibrominated **4d** might have been achieved by adding carrier bromide, but it was felt that a lower specific activity material obtained would not be optimal for protein labeling. Knight et al. reported that the Bolton–Hunter reagent could be radiobrominated (⁷⁷Br) or radioiodinated (¹³¹I) by employing the enzyme chloroperoxidase and subsequently used to conjugate with fibrinogen and serum albumin (188). Low radiochemical yields were obtained using this method.

A reagent similar to the Bolton–Hunter reagent, *N*-succinimidyl (*tert*-butyloxycarbonyl)-L-[¹²⁵I]iodotyrosine (**5**), has been reported by Assoian et al. (189). This reagent was designed to be conjugated to a protein with subsequent removal of the *t*Boc protecting group to regenerate the positive charge lost in the conjugation step. Unfortunately, the deblocking step required that the iodinated protein be dried and 99% formic acid be added for 2–4 h at room temperature or overnight at 4 °C. These conditions were satisfactory for the peptides studied, but such conditions can be expected to cause deleterious effects to most proteins.

As mentioned previously, studies at Los Alamos which involved ⁷⁷Br-labeled monoclonal antibodies using the Bolton–Hunter reagent were unsuccessful (187). This led to the application of an arylsilane intermediate for regiospecific incorporation of bromine-77 into an amine-reactive compound. Thus, *N*-succinimidyl 3-[4-(trimethylsilyl)phenyl]propionate, **7a**, was synthesized and radiobrominated to form **8a** (190). Compound **7a**, an organometallic counterpart to the Bolton–Hunter reagent, was prepared and radiobrominated efficiently using NCS and Na^{77,82}Br in MeOH/HOAc at 50 °C for 5 min. However, radioiodination did not give the desired products under the conditions studied. There were no protein conjugations carried out with **8a**.

Radiohalogenated benzoic acids have been of interest for radiolabeling proteins and have been studied by a number of different research groups. The primary reason for the use of the more difficult to radiohalogenate, nonphenolic aryl ring benzoates was to stabilize the radiohalogen to in vivo dehalogenation. Additionally, the halogenated benzoyl conjugates were thought to be cleaved to benzoic acids which, for the *m*- and *p*-halobenzoic acid derivatives, would likely be rapidly converted in vivo to the corresponding hippuric acids and cleared through the renal system (191).

The earliest reported radiohalogen labeling of a benzoic acid was conducted by Hughes and Klinenberg, who demonstrated that 4-diazobenzoic acid, **9a**, could be used to prepare 4-astatobenzoic acid, **10a** (130). Subsequently, Friedman et al. demonstrated that 4-astatobenzoic acid, **10a**, could be used to stably radiolabel bovine serum albumin (BSA) by in situ formation of the isobutyl mixed anhydride **10b** (192). The experiments provided radiochemical yields of about 12% in the conjugation step. Vaughan was able to improve the radiochemical yield to 30–35% (10% overall labeling yield) when rabbit anti-mouse thymocyte IgG was labeled with the same reagent (193). Harrison and Royle also used the same approach to label a rabbit IgG with astatine-211 (194). These

Table IV. Amine-Reactive Radiohalogenation Reagents^a

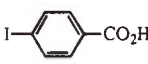
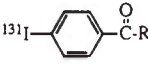
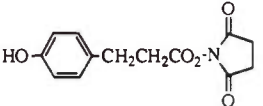
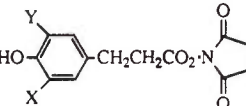
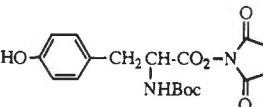
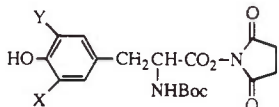
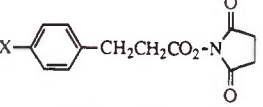
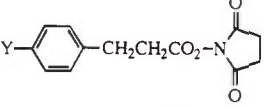
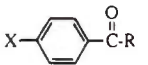
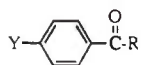
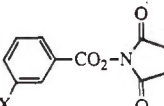
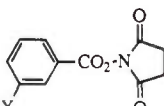
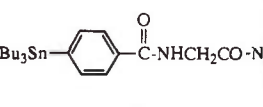
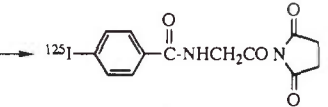
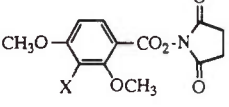
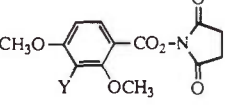
starting reagent	radiolabeled reagent (for protein conjugation)	% radiolab yield	% conjugn yield	ref
A. Activated Carboxylates				
 1	 2a: R = OH 2b: R = Cl	25-90	25-75	179
 3	 4a: X = ¹²⁵ I, ¹³¹ I, ¹²³ I; Y = H 4b: X = Y = ¹²⁵ I, ¹³¹ I, ¹²³ I 4c: X = ⁷⁷ Br; Y = H 4d: X = Y = ⁷⁷ Br	30-75 NR (to 85)	13-53 35-65	180 188
 5	 6a: X = ¹²⁵ I; Y = H 6b: X = Y = ¹²⁵ I	55-65	20-50	189
 7a: X = SiMe ₃ 7b: X = SnBu ₃	 8a: Y = ⁷⁷ Br, ⁸² Br 8b: Y = ¹²⁵ I	>80 51	NR NR	190 195
 9a: X = N ₂ ⁺ ; R = OH 9b: X = SnBu ₃ ; R = NHS 9c: X = SnBu ₃ ; R = CNP 9d: X = SnBu ₃ ; R = TFP 9e: X = SnBu ₃ ; R = PFP 9f: X = NO ₂ ; R = NHS 9g: X = NMe ₃ OTf; R = H	 10a: Y = ²¹¹ At; R = OH 10b: Y = ²¹¹ At; R = -OCO ₂ i-Bu 10c: Y = ¹²⁵ I, ¹³¹ I, ¹²³ I; R = NHS 10d: Y = ⁷⁷ Br; R = NHS 10e: Y = ²¹¹ At; R = NHS 10f: Y = ¹²⁵ I; R = CNP 10g: Y = ¹²⁵ I; R = TFP 10h: Y = ¹²⁵ I; R = PFP 10i: Y = ¹⁸ F; R = H 10j: Y = ¹⁸ F; R = OH 10k: Y = ¹⁸ F; R = NHS	NR 37-97 60-95 44-61 70-90 61-71 71-74 41-74 17-48	12-59 40-70 60-70 60-60 26 36 29	130 192-194 81 82 83 196 196 196
 11a: X = SnBu ₃ 11b: X = SnMe ₃ 11c: X = HgOAc	 12a: Y = ¹²⁵ I, ¹³¹ I 12b: Y = ²¹¹ At	85-90 67-95	33-65 39-65	79, 204, 205 80, 207
 13	 14	57	NR	195
 15a: X = Bu ₃ Sn 15b: X = Me ₃ Sn	 16: Y = ¹³¹ I	70-75	48-68	216

Table IV. (Continued)

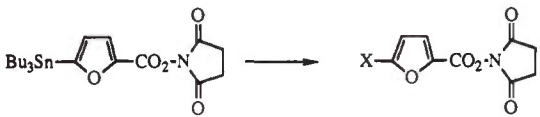
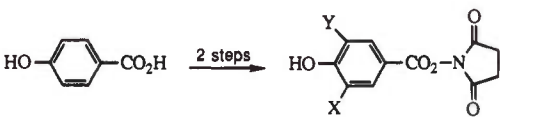
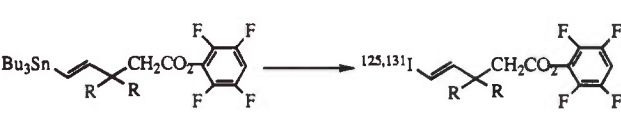
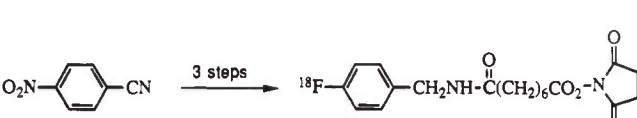
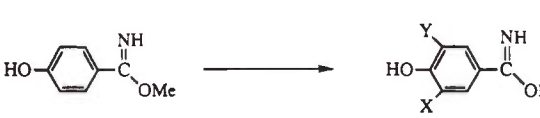
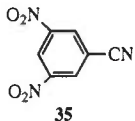
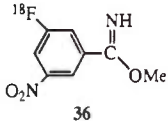
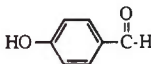
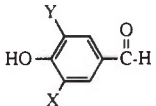
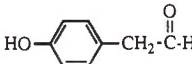
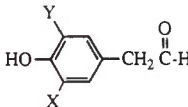
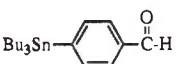
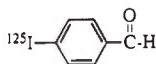
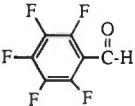
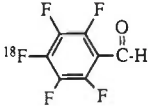
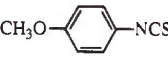
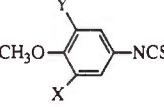
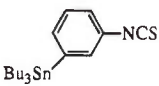
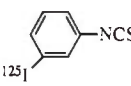
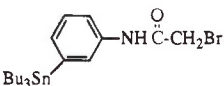
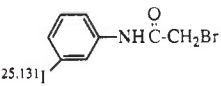
starting reagent	radiolabeled reagent (for protein conjugation)	% radiolab yield	% conjugn yield	ref
A. Activated Carboxylates (Continued)				
 17a: X = Bu₃Sn 17b: X = Me₃Sn 18: Y = ¹²⁵I, ¹³¹I		80–88	56–65	217
 19 20a: X = ¹³¹I 20b: X = ²¹¹At		70–80	38–58	218
 21 22a: X = ¹²⁵I; Y = H 22b: X = Y = ¹²⁵I		50–90	10–15	219
 23a: R = H 23b: R = CH₃ 24a: R = H 24b: R = CH₃		70–95	25–50	222
 25a: R = OH 25b: R = NHS 25c: R = NH-Protein 25d: R = OC(CH₃)₃ 26a: R = NH-Protein 26b: R = OH 26c: R = NHS		90	NR	225
 27 28		25–40	26–52	226
 29a: R = phenol 29b: R = <i>p</i>-CN-phenol 29c: R = O-TFP 30a: R = phenol 30b: R = <i>p</i>-CN-phenol 30c: R = O-TFP		60 25 29	NR NR 52	227
B. Imidates				
 31 32a: X = ¹²⁵I; Y = H 32b: X = Y = ¹²⁵I		60–80	20–30	230
 33 34		55	NR	195

Table IV. (Continued)

starting reagent	radiolabeled reagent (for protein conjugation)	% radiolab yield	% conjugn yield	ref
B. Imidates (Continued)				
 35	 36	42-45	17-30	235
C. Aldehydes				
 37	 38a: X = ^{125}I ; Y = H 38b: X = Y = ^{125}I	70-95	NR (est. 60%)	241
 39	 40a: X = ^{125}I ; Y = H 40b: X = Y = ^{125}I	>90	8-58	242
 41	 42	NR	NR	240
 43	 44	60	NR	227
D. Isothiocyanates				
 45	 46a: X = $^*\text{I}$; Y = H 46b: X = Y = $^*\text{I}$	40-55	35-45	248
 47	 48	23-55	NR	249, 250
E. Acetyl Bromides				
 49	 50	80	48-60	251
^a Where NR = not reported; NHS =  ; CNP =  ; TFP =  ; PFP = 				

investigators were able to obtain a radiochemical yield of 50% (decay corrected). While the radiochemical yield of 50% was quite reasonable, the procedures used in astatine labeling were time-consuming, taking 3 h to complete (25% At-211 decay), and employed chemical extractions which could pose a safety risk when conducted.

Studies of radiohalogenation of proteins (antibodies) at NeoRx initially involved synthesis and radioiodination of *N*-succinimidyl 4-(tri-*n*-butylstannyl)benzoate, **9b**, *N*-

succinimidyl 3-[4-(tri-*n*-butylstannyl)phenyl]propionate, **7b**, *N*-succinimidyl 4-(tri-*n*-butylstannyl)hippuric acid, **13**, and methyl 4-(tri-*n*-butylstannyl)benzimidate, **33**. Synthesis of these compounds was readily accomplished using standard techniques (195). The radioiodinations were found to be facile in all examples studied, but the stability toward ester or imide hydrolysis (under the conditions studied) was markedly different, resulting in the large variations in radiochemical yields of desired product.

Because the benzoate ester **10c** was found to be the most stable radioiodinated compound toward hydrolysis, it was chosen for further study.

A large effort to optimize the chemistry of radioiodine labeling of **9b** and conjugation of the radioiodinated **10c** to monoclonal antibodies was conducted at NeoRx (196). The *N*-hydroxysuccinimide (NHS) ester had been found to be relatively stable to hydrolysis/methanolysis in the radiolabeling medium; however, it was of interest to determine whether other active esters might improve the conjugation yields. The stannylbenzoate esters of 2-chloro-4-nitrophenol (CNP), **9c**, 2,3,5,6-tetrafluorophenol (TFP), **9d**, and pentafluorophenol (PFP), **9e**, were prepared and studied. Radioiodination reactions were conducted in methanol, ethanol, and 2-propanol at room temperature and 55 and 75 °C. The results of those studies indicated that the radioiodination was similar for all of the different esters. Conjugation studies of the radioiodinated benzoate esters **10c,f-h** were carried out. All of the ester conjugations gave similar yields, but the NHS ester conjugation yields were slightly higher than the others. Further optimization of conjugation conditions was carried out on the NHS and TFP esters **10c** and **10g**. Again, the TFP ester **10g** gave similar conjugation yields to the NHS ester **10c** under optimized conditions, however, purification of the protein conjugated with the TFP ester was more difficult due to what appeared to be an "association" of the unreacted ester with the protein. Treatment of the radiolabeled protein with a 1 M lysine or glycine solution could be used to improve the radiochemical purity the protein conjugated with the TFP ester. This treatment was not necessary for purification of the NHS ester conjugated proteins, so the final choice of ester for use with the radiohalogenated benzoate was the NHS ester. Optimization studies of the benzoate NHS conjugations included the use of borate, phosphate, and carbonate buffers in conjugation reactions at various pHs. While borate buffer (pH 9.0) gave the highest conjugation yields (to 73%), bicarbonate/carbonate buffer (pH 8.5) was chosen due to concerns for borate buffer in an injectable preparation. Bicarbonate buffer gave good conjugation yields (to 60%). Indeed, under optimized conditions, where 1-mg quantities of protein at 3–5 mg/mL concentration were labeled, conjugation yields of 60–80% have been obtained using bicarbonate buffer.

The benzoate NHS has been used extensively for radioiodinated monoclonal antibodies in preclinical studies (81, 197–201). The studies have conclusively shown that free radioiodide is not released in vivo when the *p*-iodobenzoyl (PIB) labeling method is used, in contrast to antibodies that were radioiodinated by the direct labeling method. Radioiodinated monoclonal antibodies labeled with **10c** (iodine-123,125,131) have also been used in clinical studies (202, 203). Studies involving the use of the stannylbenzoate NHS ester **9b** with other radiohalogens have demonstrated that it can be used to radiolabel proteins with bromine-77 (82) and astatine-211 (83) as well.

Similar to our studies, Zalutsky and his co-workers have conducted a large number of studies on the use of *m*-halobenzoic acid conjugates of monoclonal antibodies (79, 80, 204–210). They have found iodine-125, iodine-131, or astatine-211 could be incorporated efficiently into the meta position of benzoic acid with either the tri-*n*-butylstannyl group, compound **11a** (*m*-BuATE), or the trimethylstannyl group, compound **11b** (*m*-MeATE). Higher yields were consistently observed using **11b** in the astatination reactions. The in vivo stability of the

radioiodinated **12a** was very good and the investigators have reported that they obtained enhanced tumor localization (206) and improved therapeutic efficacy (210) over the same antibodies directly labeled. Vaidyanathan and Zalutsky compared the stability of the radioiodine label on an antibody toward in vivo dehalogenation when it was labeled directly, labeled with **12a**, and labeled with the Bolton–Hunter reagent, **4a** (211). They found that the stability of the benzoyl label **12a** was highest, having about twice the stability of the Bolton–Hunter label. Both of the conjugates were considerably more stable than the direct label on the protein. Zalutsky and co-workers chose the meta isomer over the para isomer of benzoic acid, as they felt that it would be less susceptible to nucleophilic displacement of radioiodine. A comparison of the in vivo stability of coinjected, dual-labeled *p*-iodobenzoate **10c** and the *m*-iodobenzoate **12a** on an intact antibody provided data that suggested that the meta isomer was more stable (208). Comparative studies of coinjected, dual-labeled monoclonal antibody Fab fragment at NeoRx provided data suggesting that the radiolabels had essentially the same in vivo stability, but a large difference in the kidney retention of radioactivity was noted for radiolabeled antibody Fab fragments (197). Subsequent to those studies, Williams et al. compared the *m*- and *p*-iodobenzoyl conjugates with directly labeled antibody and found that the two conjugates were comparable, but an increased liver retention was noted for the meta isomer (212). Two other approaches to the preparation of **12a** have been described (213). A very complex approach involved radioiodination of aminobenzoic acid, removal of the amine through diazotization/decomposition, and preparation of NHS ester. As this procedure involves several steps with radiolabeled materials, it is quite undesirable. The second method of preparation of **12a** by the same investigators was the use of an organomercury intermediate, **11c**. The tetrafluorophenyl ester of *m*-iodobenzoic acid has also been prepared by Hanson et al. (214).

Vaidyanathan and Zalutsky have reported an investigation of the use of *N*-succinimidyl 4-¹⁸F-fluorobenzoate, **10k**, for radiofluorination of monoclonal antibodies (215). An initial attempt to produce **10k** via nucleophilic displacement of an aromatic nitro group by [¹⁸F]fluoride on *N*-succinimidyl 4-nitrobenzoate, **9f**, was unsuccessful. However, fluorobenzaldehyde **10i** was obtained by [¹⁸F]-fluoride substitution on 4-formyl-*N,N,N*-trimethylanilinium triflate, **9g**, in 40–80% radiochemical yield. The radiofluorinated benzaldehyde **10i** was then oxidized to the corresponding benzoic acid **10j** in 55–80% radiochemical yield and esterified to produce the desired NHS ester **10k** in 31–89% yield (depending on time allowed). Total synthesis time was 100 min with about 25% (decay corrected) radiochemical yield (calculated 17–48%). Conjugation to a monoclonal antibody was achieved in good yield.

Zalutsky has also described several other new compounds for use as radiohalogenated conjugates of proteins (216–219). To incorporate a radioiodine into an aromatic ring which would have the halogen sterically blocked, the dimethoxy congener of *N*-succinimidyl *m*-iodobenzoic acid, **16**, was prepared and evaluated in vivo. *N*-Succinimidyl 2,4-dimethoxy-3-(tri-*n*-butylstannyl)benzoate, **15a** (220), and the corresponding trimethylstannyl compound, **15b** (216), were synthesized and radioiodinated. The in vivo data indicated that there was extensive in vivo deiodination. This result suggests that demethylation may be

occurring in vivo, resulting in an iodinated hydroxyphenol, which might be expected to undergo rapid deiodination.

Another radioiodinated conjugate that has been described by Zalutsky et al. is a pyridine derivative, *N*-succinimidyl 5-[¹³¹I]-3-pyridinecarboxylate, **18** (217, 218). The pyridine derivative was studied because its structure is dissimilar to the compounds, iodotyrosine and thyroxine, recognized by deiodinase enzymes. A similar protein conjugate, 5-iodopyridine-2-carboximide, has been described for preparing (nonradioactive) heavy-atom derivatives of protein (221). In the study of Zalutsky et al., *N*-succinimidyl 5-(tri-*n*-butylstannyl)-3-pyridinecarboxylate, **17a**, and *N*-succinimidyl 5-(trimethylstannyl)pyridinecarboxylate, **17b**, were synthesized and studied in radioiodination reactions. Radioiodinations required elevated temperatures (60–65 °C), but good radiochemical yields were obtained. *N*-Chlorosuccinimide (NCS) and *tert*-butyl hydroperoxide were studied as oxidants. The best yields were obtained with the trimethylstannyl intermediate **17b**, using NCS as the oxidant at 60–65 °C for 5 min. Conjugation reactions were conducted at pH 8.5 for 15 min and good yields were obtained. The iodinated pyridinecarboxylate (nicotinic acid) conjugates were found to be very stable toward in vivo deiodination.

A preliminary account of a furan ring conjugate, *N*-succinimidyl 5-(tri-*n*-butylstannyl)furan-2-carboxylate, **19**, has been reported by Zalutsky et al. (218). Studies have shown that this reagent can be radioiodinated to give **20a** in good yield. It has also been labeled with astatine-211 to prepare **20b**. The in vivo data from the radioiodinated furancarboxylate **20a** labeled antibody suggest that it is not as stable toward deiodination as the coinjected *m*-iodobenzoate **12a** labeled antibody.

In another investigation, Zalutsky et al. studied the use of *N*-succinimidyl 4-hydroxy-3-[¹³¹I]iodobenzoate, **22**, as a conjugate for monoclonal antibodies (219). This investigation was conducted to ascertain whether there was a difference in the in vivo stability between **22** and the Bolton–Hunter reagent, **3**, where there is a difference of two methylenes in the structures. Compound **22** was prepared from 4-hydroxybenzoic acid, **21**, followed by esterification. High yields for the radioiodination and esterification steps were obtained. No discussion of the possible mono- and diiodo products **22a** and **22b** was given. Conjugation yields were low compared with those of other benzoate reactions. There were no differences in thyroid activity (a measure of stability) at 24 h for coinjected antibodies labeled with hydroxybenzoyl-[¹³¹I]**22** and the similar nonphenolic [¹²⁵I]**12a**. However, at later time points in the study differences in thyroid activity were observed which suggested that the hydroxy compound was less stable than the nonphenolic counterpart.

Two vinyl iodo compounds, 2,3,5,6-tetrafluorophenyl 5-[¹²⁵,¹³¹I]iodo-4-pentenoate, **24a**, and 2,3,5,6-tetrafluorophenyl 3,3-dimethyl-5-[¹²⁵,¹³¹I]iodo-4-pentenoate, **24b**, were prepared at NeoRx and studied as conjugates for monoclonal antibodies (222). The vinyl tri-*n*-butylstannyl derivatives **23a** and **23b** were synthesized from the corresponding acetylenic carboxylic acid methyl esters using hydrostannylation, followed by hydrolysis/reesterification with tetrafluorophenol. A more direct method of synthesizing **24a** involving stannylation of the tetrafluorophenyl ester of the alkyne has been described in a preliminary report by Hanson et al. (214). In our studies, esters **23a** and **23b** were radioiodinated using NCS as the oxidant, which resulted in good radiochemical yields of **24a** and **24b**. In vivo studies with a radiolabeled antibody Fab fragment indicated that the stability of the vinyl iodide in **24a** was not much higher than that obtained by direct

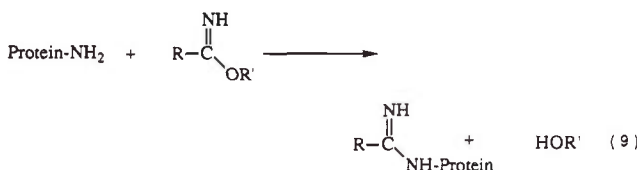
labeling with chloramine-T. However, the dimethyl vinyl iodo ester **24b** labeled Fab was found to have an intermediate stability toward dehalogenation compared with chloramine-T-labeled Fab. It had been anticipated that the dimethylpentenoic acid would be blocked from undergoing normal fatty acid catabolism (β -oxidation), as previously observed for dimethyl fatty acid derivatives (223, 224). With these limited studies it was concluded that vinyl iodides are not the conjugates of choice for protein labeling.

Reagents for radiofluorination of proteins which are conjugated by active esters have also been reported (225–227). For example, the NHS ester of α -bromo-*p*-toluic acid, **25b**, has been used to conjugate it to small peptides and amine-containing compounds. Once conjugated to the amine-containing peptide to give **25c**, the benzyl bromide was replaced with fluoride in refluxing acetonitrile in the presence of excess tetraalkylammonium fluoride, yielding radiofluorinated **26a**. This method would not be practical for fluorination of larger proteins. However, the investigators described another route for protein conjugation which uses the [¹⁹F]fluoro NHS ester **26c**. This route involves the fluorination of *tert*-butyl ester of α -bromo-*p*-toluic acid, **25d**, with tetrabutylammonium fluoride in acetonitrile, and has been accomplished in high yield (225). Subsequent cleavage of the *tert*-butyl group was accomplished in 10 min with exposure to neat trifluoroacetic acid and the NHS ester could be prepared in the usual manner. The authors did not report radiofluorinations using this reagent.

Zalutsky et al. have described the use of 4-[¹⁸F]-fluorobenzylamine, produced from the reaction of tetrabutylammonium [¹⁸F]fluoride with *p*-nitrobenzonitrile, **27**, and subsequent reduction with lithium aluminum hydride (226). The fluorobenzylamine was then reacted with disuccinimidyl suberate (DSS) to form the radiofluorinating reagent **28** in situ. It was found that the protein coupling yields could be improved by 15–40% when **28** was purified by HPLC. This difference in yield is believed to be brought about from excess DSS competing for amines on the protein. The synthesis times were long, 80–90 min, but good overall yields were obtained. Indeed, a preliminary study of the use of this reagent conjugated to an antibody and studied in a canine myocardial infarct model has been reported (228).

A preliminary investigation of the use of pentafluorobenzoate esters for radiofluorination of proteins has been reported (227). Pentafluorophenyl groups will undergo exchange (primarily in the position para to the non-fluorine group) with [¹⁸F]fluoride. The tetrafluorophenyl ester of pentafluorobenzoate, **29c**, was found to react rapidly (16 min) and was stable under the exchange conditions (tetrabutylammonium [¹⁸F]fluoride, heat, polar solvents). The product of the exchange reaction gave an overall labeling yield of 15% when reacted with human serum albumin. While reasonable yields were obtained, it should be noted that this labeling method suffers from the fact that low specific activity labeling would be expected, and this may cause problems with its general application.

2. Imidate Esters. Imidate esters react with protein amines to form amidine bonds as shown in eq 9. An



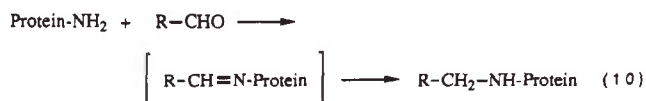
important characteristic of the amidine bond is that it is protonated at physiological pH, resulting in retention of the charge of the original amine. Thus, unlike the formation of amide bonds, the positive charge initially carried by the lysine amine is retained. The imidate esters are more resistant to hydrolysis than many active esters, particularly *N*-hydroxysuccinimide esters, but the reaction rates for aminolysis of the methyl ester is also slower, resulting in longer overall reaction times. Conjugation reactions of imidate esters are pH dependent, the extent of which has been shown to be a function of the amine and imido ester (229).

An imidate ester of phenol which was used as a protein conjugate for radioiodination was described by Wood, Wu, and Gerhart (230). They synthesized methyl *p*-hydroxybenzimidate, 31, and prepared the mono- and di[¹²⁵I]-iodinated derivatives 32a and 32b in high specific activity. The overall labeling yields appear to be comparable to or slightly lower than those of the Bolton-Hunter reagent. This reagent (Wood's reagent) was reported to have the same advantages of mild protein labeling conditions as the Bolton-Hunter reagent, with the added advantage of retention of the positive charge on the amidine functionality. However, the authors point out the problem of ionization of the phenol when it is diiodinated. Thus, the net overall charge would be neutral, not retention of a positive charge. The Wood's reagent has been used to radiolabel proteins (231-233) and cell surface proteins (234).

An organometallic benzimidate, methyl *p*-(tri-*n*-butylstannyl)benzimidate, 33, was synthesized at NeoRx (195). This compound was radiolabeled efficiently (>90%) to give two radiolabeled products, but the desired iodo compound was obtained in only 55% yield. The other radioiodinated product was not identified but, due to its similar HPLC retention characteristics, was thought to be a radioiodinated phenyl compound. No further studies on the optimization of the radioiodination or on the conjugation with a protein have been done. However, the radioiodinated compound 34 remains of interest as it retains the positive charge without concern of an ionizable hydroxyl group being present.

A radiofluorinated benzimidate, 3-[¹⁸F]fluoro-5-nitrobenzimidate, 36, has been investigated for use in protein labeling by Kilbourn et al. (235). In conjugation reactions, effects of protein concentration, reaction time, and pH were studied. It was found that the conjugation yields did not increase appreciably above pH 7.5 but were increased dramatically with higher concentrations of protein. Conjugations were conducted at 47 °C, pH 8.0, for 1 h.

3. Aldehydes. Protein amines react with aldehydes to form imines (Schiff bases) which can subsequently be reduced with NaBH₄ (236-238) or NaBH₃CN (239) to form secondary amines as shown in eq 10. The reductions are



generally carried out at pH 9 when NaBH₄ is employed, but can be conducted as low as pH 5 for labeling of α -amino groups when the more stable NaBH₃CN is employed.

Conjugates containing phenolic rings for radiohalogenation and aldehyde functionalities for conjugation to proteins have been developed. Citing a need to preserve the nucleophilicity of conjugated amines of proteins for biological activity, particularly on small peptides, Su and

Jeng radioiodinated 4-hydroxybenzaldehyde, 37, and investigated conjugation of the radioiodinated product, 38, to glycine and lysine derivatives by reductive amination with sodium cyanoborohydride (240). Conjugation/reduction was conducted for 20 h at 37 °C. Two major products were observed, which the authors felt may have been mono- and di-*N*-alkylated products. Another explanation may be that the products obtained were the mono- and diiodinated conjugates of a monoalkylated product.

Panuska and Parker reported that protein labeling with 38 gave low yields, so they investigated the application of a similar reagent, (4-hydroxyphenyl)acetaldehyde, 39, for protein labeling (241). This reagent was used because the aldehyde group would be expected to be more reactive with protein amines due to its separation from the aromatic ring by the methylene group. In their studies, the enzyme β -galactosidase was reacted with radioiodinated 40 and NaBH₃CN at 4 °C for 24 h with no loss of enzymatic activity. The investigators found that conjugation could be accomplished over a range of pHs, allowing one to select an appropriate pH to minimize damage to the protein. They reported that "efficient incorporation" of radiolabel into the protein was obtained even at low concentrations of protein (e.g. 40 μ g/mL).

Nonphenolic radioiodinated aldehydes were prepared at NeoRx (242). An arylstannane, *p*-(tri-*n*-butylstannyl)benzaldehyde, 41, was prepared and radioiodinated. Synthesis of 41 was accomplished by stannylation of *p*-bromobenzyl alcohol using (Bu₃Sn)₂/Pd(PPh₃)₄ in toluene, followed by oxidation of the alcohol with pyridinium chlorochromate in CH₂Cl₂. (The phenacetaldehyde was also prepared in this manner, but a low yield was obtained and it was relatively unstable, so further studies were not pursued.) Radioiodination of 41 to prepare *p*-[¹²⁵I]-iodobenzaldehyde, 42, was facile giving radiochemical yields of >90%. Conjugations were conducted with 42 at pH 8.5-9.5 with various molar ratios of the reductant NaBH₃CN. Conjugation yields ranging from 8 to 58% were obtained after a 24-h reaction period.

A preliminary investigation of the use of pentafluorobenzaldehyde, 43, for radiofluorination to yield 44 and reductive alkylation to conjugate 44 with proteins has been reported by Herman et al. (227). The authors found that radiofluorination could be accomplished by exchange with tetrabutylammonium fluoride in DMSO to give 60% (decay corrected) yield within 30 min. Reductive alkylation of HSA using NaBH₄ in H₂O/DMSO for an additional hour gave an overall yield of 15% (decay corrected). Their brief report also indicated that when NaBH₃CN was used as the reductant, *in vivo* hydrolysis of the labeled aldehyde (imine) occurred.

4. Isocyanates and Isothiocyanates. Amines react with isocyanates (X = O) to form urea bonds (243) and isothiocyanates (X = S) to form thiourea bonds as depicted in eq 11. The isothiocyanate moiety is more stable toward

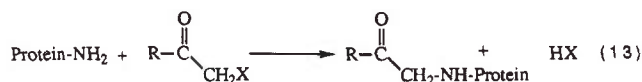


hydrolysis and is most often used for protein conjugation. Many conjugates of metal chelates have used isothiocyanates for conjugation (244-247), but few radiohalogen labeling reagents have been developed using this functionality.

The use of *p*-methoxyphenyl isothiocyanate, 45, as a radioiodination reagent has been described in a preliminary report by Dewanjee et al. (248). Radioiodination of 45 yielded 46 with labeling efficiencies of 40-55%. Conju-

gation of 46 was achieved in radiochemical yields of 35–45%. In a recent publication, Ram and Buchsbaum have described preparation of 3-iodophenyl isothiocyanate, 48, for radioiodination of monoclonal antibodies (249). These investigators used a stannyl intermediate, 3-tri-*n*-butylstannylphenyl isothiocyanate, 47, to prepare the radioiodinated 48 in moderate to high yields. In a related preliminary report, these investigators reported a low yield for conjugation of 48 to a monoclonal antibody (250), but on the basis of other conjugations containing isothiocyanate functionalities, it is likely that the conjugation yields can be improved.

5. Activated Halides and Maleimides. Nucleophilic amines will react with compounds such as benzyl halides, acetyl halides, and maleimides to form alkylated amines as depicted in eqs 12–14. These types of compounds are



Where: X = I, Br, Cl, Tosylate, etc.



most often thought of as reagents for conjugations with thiol groups; however, depending on pH and availability of amines, conjugations with protein amines rather than thiol groups might be expected. Therefore, the conditions for conjugation of these groups must be carefully controlled.

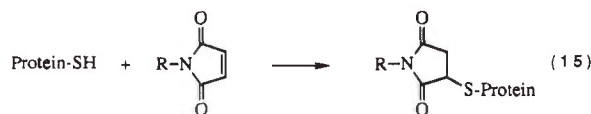
An example of conjugation with an acyl bromide, (3-[¹²⁵I, ¹³¹I]iodophenyl)bromoacetamide, 50, to an antibody via amino groups has been reported by Khawli et al. (251). The radioiodination reagent 50 was prepared from [3-(tri-*n*-butylstannyl)phenyl]bromoacetamide, 49. The investigators conducted the reactions at pH 9.5 for 30 min and obtained good conjugation yields.

B. Sulfhydryl-Reactive Conjugates. The second most utilized functional group on a protein for formation of conjugates is the sulfhydryl (thiol) group present on cysteine residues. Thiols are nucleophiles like amines and will react with many of the functional groups described for conjugation of amines. Reaction with activated carboxylates yields thioesters which are themselves activated esters, and therefore are inherently unstable. However, reactions that form thioethers provide stable conjugates. Thiols can be reacted selectively in preference to the amines on a protein because they are better nucleophiles, particularly if the amines are protonated through adjustment of the pH (7 or less). Indeed, the most facile and highest yielding radiohalogenation reactions conducted in our laboratory have involved thiol conjugations.

There is a large amount of literature on the modification of thiols on proteins (252). Proteins which do not contain native thiols can be reacted with compounds such as 2-iminothiolane (which is commercially available as Traut's reagent) to generate them (253–255). Proteins lacking free sulfhydryl groups but containing disulfide bridges (e.g. antibodies) can be treated with thiol-containing reagents such as β-mercaptoethanol (256) or dithiothreitol (DTT, Cleland's Reagent) to produce free thiols (albeit transiently). These thiol-generating reagents are shown

in Table III. Thiol-reactive radiohalogenation reagents that have been studied are listed in Table V. A brief discussion of the chemistry involved in the thiol reactive conjugates is given in the following text.

1. Maleimides. Maleimide-containing compounds react with thiol groups to form thioethers as shown in eq 15.



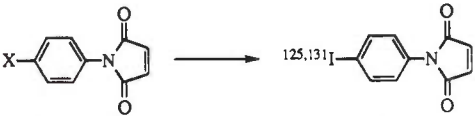
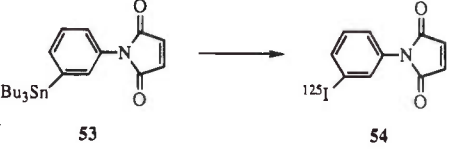
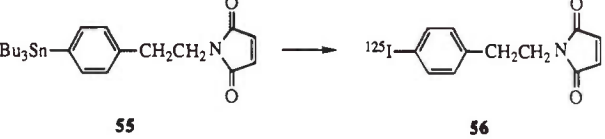
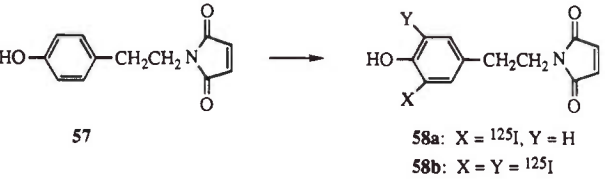
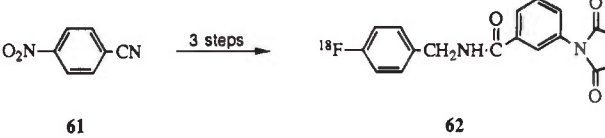
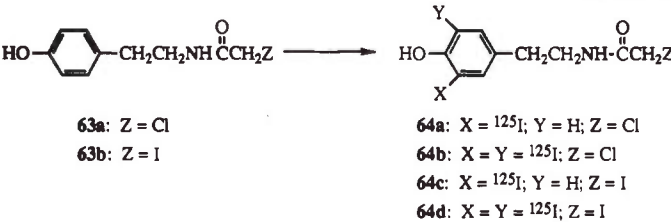
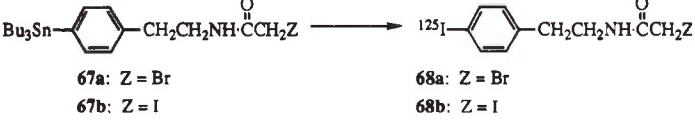
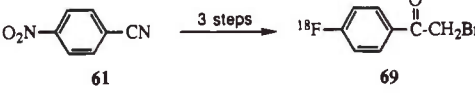
Conjugation reactions are generally conducted at pH 7 or below and are complete within a few minutes.

A phenylmaleimide, *N*-(*p*-[¹²⁵I]iodophenyl)maleimide, 52, has been prepared and evaluated in protein conjugation reactions by Srivastava et al. (257). These investigators used the arylmercury intermediate *N*-[*p*-(acetylmercuric)-phenyl]maleimide, 51a, for radioiodination to prepare 52. Relatively low radioiodine labeling yields were obtained through a sequence of reaction/extraction/purification conducted over a 60-min period, with a maximum radiolabeling yield of 35%. Conjugation of the radioiodinated 52 also gave low radiochemical yields. Although a figure (Figure 2) in their report indicates that the reagent was conjugated by reaction of a free sulfhydryl group, it is unclear that sulfhydryls were present to react. Unless the antibody used had an available sulfhydryl for reaction with 52, it must be surmised that protein amines reacted in the conjugations.

Studies at NeoRx employed the arylstannane *N*-[*p*-(tri-*n*-butylstannyl)phenyl]maleimide, 51b, to prepare radioiodinated 52 (258). Much higher radiochemical yields were obtained using the arylstannane 51b than was reported for the arylmercury intermediate 51a. The maleimide ring of 51b was readily opened through alcoholysis with either methanol or ethanol to form the maleamidic ester. This ring opening is a common side reaction of *N*-phenylmaleimides and is due to the conjugation of the nitrogen lone-pair electrons with the phenyl group (259). It was found that radioiodination reactions of 51b could be carried out without maleimide ring opening when 2-propanol/15% HOAc was used as the solvent. In this solvent mixture, the reactions were much slower than in the usual methanol/1% HOAc mixtures, but gave average radiochemical yields of 65%. The NeoRx studies used intact monoclonal antibodies and their Fab fragments which were pretreated with DTT to produce sulfhydryls for conjugation. Very careful handling of the DTT-treated antibodies provided conjugation reactions that were fast and gave high yields. The radioiodinated methyl maleimilate derivative of 52 was (purposefully) produced in one experiment to ascertain if this compound would also conjugate to a DTT-treated antibody. It seemed reasonable that α,β-unsaturated amido compound might undergo a Michael type addition similar to maleimides. Indeed, this was the case with 80% conjugation yield being obtained at pH 6.0.

Khawli et al. have recently reported the preparation of *N*-(*m*-[¹²⁵I]iodophenyl)maleimide, 54 (260). The arylstannane *N*-[*m*-(tri-*n*-butylstannyl)phenyl]maleimide, 53 was prepared and radioiodinated in good yields to give 54. The investigators used a biphasic reaction mixture (H₂O/CH₂Cl₂) in the radioiodination step to effectively minimize the ring opening hydrolysis reaction. Conjugation of 54 with rabbit IgG and bovine serum albumin was accomplished by using the heterobifunctional coupling reagent *N*-succinimidyl 3-(2-pyridylthio)propionate (SPDP) for introduction of latent sulfhydryls. Reduction with dithio-

Table V. Thiol-Reactive Radiohalogenation Reagents^a

starting reagent	radiolabeled reagent (for protein conjugation)	% radiolab yield	% conjugn yield	ref
A. Maleimides				
 51a: X = HgOAc 51b: X = SnBu₃ 52		10–35 56–88	11–25 80–95	257 258
 53 54		73	40–80	260
 55 56		59–100	85	261
 57 58a: X = ¹²⁵I, Y = H 58b: X = Y = ¹²⁵I		NR	NR	262
 59 60		15	NR	263
 61 62		10	50	263
B. Acyl Halides				
 63a: Z = Cl 63b: Z = I 64a: X = ¹²⁵I; Y = H; Z = Cl 64b: X = Y = ¹²⁵I; Z = Cl 64c: X = ¹²⁵I; Y = H; Z = I 64d: X = Y = ¹²⁵I; Z = I		44–50 80–90	43–58 75–85	264 265
 65 66		92–96	NR	266
 67a: Z = Br 67b: Z = I 68a: Z = Br 68b: Z = I		81–84	20–84	258
 61 69		28–40	20–30	235

^a NR = not reported.

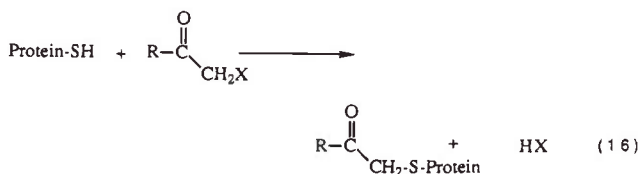
erythritol (DTE) resulted in conversion to the free sulfhydryl for conjugation with **54**. In the conjugation reaction, pH of 6.0–7.5 was found to be optimal. Sulfhydryls were also generated by treatment of intact antibody with DTE at pH 8. Interestingly, conjugation of **54** with the reduced disulfides prepared by the latter method were found to be pH sensitive, with the higher yields being obtained at pH 6.0 than obtained at pH 7.5.

As an alternative to *N*-phenylmaleimide **51b**, the phenethyl counterpart, *N*-[4-(tri-*n*-butylstannyl)phenethyl]maleimide, **55**, was synthesized and studied (261). Radioiodination of **55** could be conducted in the usual MeOH/1% HOAc solvent mixture to give very good radiochemical yields. The radiolabeling reaction was routinely conducted over 15 min, but it is likely that it was complete much sooner. Conjugation of the radioiodinated phenethylmaleimide **56** to DTT-treated antibody gave an average coupling yield of 85% in 20 min at room temperature.

A preliminary investigation of the synthesis and radioiodination of a tyramine maleimide derivative, *N*-[2-(3'-[¹²⁵I]iodo-4'-hydroxyphenyl)ethyl]maleimide, **58**, has been reported by Srivastava et al. (262). The investigators have reported radioiodinating **57** to produce the monoiodo maleimide **58a**. It is likely that they also produced some of the diiodo maleimide **58b** on the basis of other investigators' studies of radioiodinations of phenolic compounds. The investigators reported that a low thyroid uptake was obtained, indicating low in vivo deiodination, but it is unclear from the brief report what thyroid concentrations were obtained.

Preliminary studies of the use of two different maleimides for fluorine-18 labeling of antibodies have been reported by Shiue et al. (263). A phenylmaleimide, *N*-(*p*-[¹⁸F]fluorophenyl)maleimide, **60**, was prepared in four chemical steps from 1,4-dinitrobenzene. Preparation of **60** involved fluoride displacement of a nitro group on the aryl ring, followed by reduction of the remaining nitro group to an amine, condensation of the amine with maleic anhydride, and dehydration/ring closure. This process took 100 min from production of the fluorine-18 (from end-of-bombardment, EOB). The second fluorine-18-labeled maleimide, *m*-maleimido-*N*-(*p*-[¹⁸F]fluorobenzyl)-benzamide, **62**, was prepared in three steps from *p*-nitrobenzonitrile, **61**. In their preparation, the investigators displaced the nitro group with a fluoride and then reduced the nitrile to a fluorobenzylamine. The benzylamine was reacted with a cross-linking reagent, *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS), alleviating the need to prepare the maleimide. This synthesis took 70 min, but the reported yield was less than that for preparing **60**. The fluorine-18-labeled benzyl MBS derivative **62** was conjugated to rabbit IgG in 50% yield.

2. Acetyl Halides. Acetyl halides (α -halo ketones) are readily displaced by good nucleophiles such as thiol groups to form thioethers as shown in eq 16.



The preparation and radioiodination of *N*-(chloroacetyl)tyramine, **63a**, has been reported by Holowka (264). Initially, the investigators attempted to prepare the diiodo derivative **64b** by reaction with radioiodine containing 3 times the carrier sodium iodide, but they obtained a 1.7

mol of I/mol of *N*-(chloroacetyl)tyramine ratio of iodine in the product. This result and their observation of "the appearance of at least two distinct spots" by thin-layer chromatography suggests that they had a mixture that also contained the monoiodo compound **64a**. In later experiments they used no-carrier-added sodium [¹²⁵,¹³¹I]-iodide. DTT was used to produce sulfhydryl groups on the proteins studied. In the initial experiments, a large fraction of the labeled proteins were obtained as high molecular weight aggregates, which may have been formed from disulfide cross-linking. The investigators indicated that some of the radioiodinated tyramine derivative was nonspecifically bound to the protein.

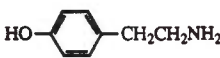
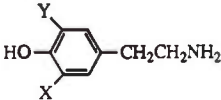
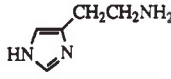
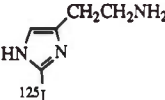
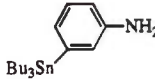
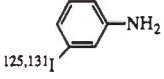
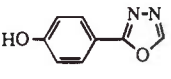
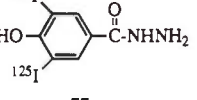
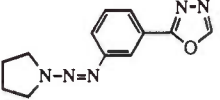
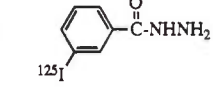
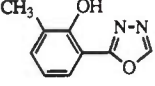
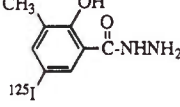
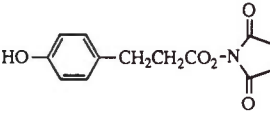
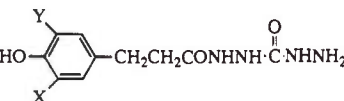
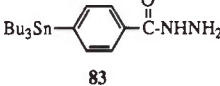
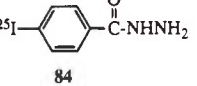
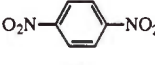
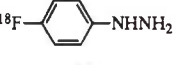
The rate of reaction of the chloroacetyl group with sulfhydryls is quite slow. Wyeth and Douglas reported obtaining higher conjugation yields with the iodoacetyl-derivatized tyramine **63b** (265). They also reported obtained much higher radioiodination yields. By reaction of the radioiodinated product **64c** (and **64d**?) with dithiothreitol (DTT) it was estimated that less than 5% of the radioiodine had exchanged with the iodoacetamido group.

In studies at NeoRx, the bromoacetyl derivatives of *p*-(tri-*n*-butylstannyl)aniline, **65**, and *p*-(tri-*n*-butylstannyl)phenethylamine, **67a**, were prepared (266). Initial studies were carried out with the bromo derivatives because it was felt that iodine exchange would occur with the iodoacetyl derivatives. While the bromo derivatives radiolabeled very well, their conjugation to DTT treated antibodies was quite slow. Conjugation with amines might have been conducted with these reagents as was done by Khawli et al. (251) with their similar reagent, **50**, but our goal was to develop a sulfhydryl conjugation reagent. Thus, to obtain higher conjugation yields, the iodoacetyl derivative of the phenethylamine **67b** was prepared and its radioiodination was studied (258). Very good radiochemical yields were obtained with no apparent exchange of the iodoacetyl iodide when the oxidant NCS was added to the radioiodide prior to adding the aryltin **67b**. Varying conjugation yields were obtained with radioiodination **68b** and were highest at pH 8, where the reaction of amines may effectively compete. The in vitro stability of the labeled proteins was found to be high, but because the reactions of thiols with the phenethylmaleimide **56** were much faster at lower pHs, no further studies with these agents were conducted.

Kilbourn et al. (235) have described the preparation of a radiofluorination reagent that contains an bromoacetyl group, 4-[¹⁸F]fluorophenacyl bromide, **69**. They were able to obtain good radiochemical (isolated) yields in a synthesis time of 75 min even though there are three chemical steps from the *p*-nitrobenzonitrile, **61**, starting material. In reactions conducted at 47 °C, pH 8.0, for 1 h, very good conjugation yields were obtained (>95%) when high concentrations of protein were used. When conducted at room temperature for 1 h, conjugation with fibrinogen was accomplished in 20–30% radiochemical yield.

C. Carbohydrate-Reactive Conjugates. Glycoproteins are important constituents of biological systems (267). Some proteins, such as monoclonal antibodies, have carbohydrate groups attached to specific regions of their structure, such as the "hinge region" (268) or the *Fc* region" (269). The fact that the carbohydrates are located at distinct areas of the antibody structures, away from the biologically active binding site, makes these functionalities attractive for "site-specific" conjugation of chemical species (270). A method of conjugation can be provided by oxidation of the oligosaccharide moieties to form reaction aldehyde functionalities on the glycoprotein molecules.

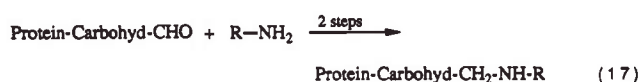
Table VI. Oxidized Carbohydrate-Reactive Radiohalogenation Reagents^a

starting reagent	radiolabeled reagent (for protein conjugation)	% radiolab yield	% conjugn yield	ref
A. Amines				
 70	 71a: X = ¹²⁵ I, Y = H 71b: X = Y = ¹²⁵ I	NR	NR	
 72	 73	70-90	NR	273
 74	 75	90	54-70	251
B. Acylhydrazines				
 76	 77	46	NR	274
 78	 79	12-30		275
 80	 81	80	25-40	276, 277
 3	 82a: X = ¹²⁵ I, Y = H 82b: X = Y = ¹²⁵ I	60-90	NR	278
 83	 84	20-40	NR	279
C. Phenylhydrazines				
 85	 86	NR	NR	280

^a NR = not reported.

Oxidation of carbohydrate moieties on antibodies is generally carried out with sodium periodate. The use of oxidized carbohydrates has permitted the addition of more moles of reagents per antibody without affecting the immunoreactivity than could be obtained by using lysines or aspartic and glutamic acid side chains in the preparation of immunoconjugates (271). A listing of radiohalogenated reagents which have been used (or conceived) for radiolabeling proteins via oxidized carbohydrates is given in Table VI. A discussion of the chemistry involved is given in the following text.

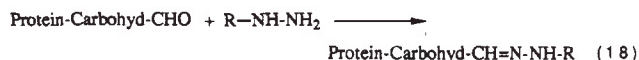
1. *Amines.* Standard condensation reactions of aldehydes with amines can produce imines. However, this reaction is reversible, so a second step involving reduction of the imine in situ must be conducted to form a stable attachment. The overall conjugation reaction of amines with oxidized glycoproteins is shown in eq 17.



A number of radiohalogenated amine-containing compounds have been prepared which could potentially be used to radiolabel proteins on oxidized carbohydrate moieties. Tyramine, 70, has been used in reductive aminations of cellobiose (272). Although carbohydrate adducts have generally been prepared prior to radioiodination, it should be possible to radioiodinate tyramine to prepare the mono- or diiodo derivatives 71a and 71b. On the basis of previous studies of radiohalogenations, the reactions will likely be better if the primary amine is protected as an amide or carbamate (e.g. tBoc group). Another amine that has potential in oxidized carbohydrate labeling is histamine, 72 (273). Radioiodinated histamine, 73, is commercially available (NEN Research Products/Du Pont) and has been used to radiolabel a number of compounds.

The preparation and conjugation of a radioiodinated iodoaniline with carbohydrate-oxidized monoclonal antibody has been described by Khawli et al. (251). These investigators prepared *m*-[¹²⁵I]₁₃₁I]iodoaniline, 75, from the corresponding arylstannane, 74. An antibody was oxidized with NaIO₄ at pH 5.6 for 1 h at 4 °C and was reacted with 75 at a 1:1 molar ratio. The immunoreactivity of the radioiodinated antibody conjugate was decreased by 20% from a control, which they felt was due to damage from exposure to sodium periodate.

2. *Hydrazines*. Another amine reaction with aldehydes, that of hydrazines, produces a hydrazone derivative as shown in eq 18, which is stable to hydrolysis (274). In



reactions of oxidized carbohydrates (with excess hydrazine derivative) 2 equiv of the hydrazine derivative can react with the α -hydroxy aldehydes to form osazones.

A number of radiohalogenated benzoylhydrazine derivatives have been prepared. Heindel et al. have reported the use of a protective oxadiazole group as a synthon for the acylhydrazine group (275). They employed the synthon for acylhydrazine since iodination of *p*-hydroxybenzoic acid hydrazide failed to provide the desired iodinated (radioiodinated) compound. Iodination and radioiodination of 2-(4'-hydroxyphenyl)-1,3,4-oxadiazole, 76, was conducted with carrier-added iodide to prepare the diiodo derivative. The phenyloxadiazole was treated with refluxing concentrated HCl/THF for 3 h to generate the benzoylhydrazine 77, which was reacted in situ with *p*-(dimethylamino)benzaldehyde for 2 h at reflux to form the benzoylhydrazone for characterization. In a later publication (276), the authors used a triazine intermediate, 2-[3'-(3',3'-(1,4-butanediyl)triazeno]phenyl]-1,3,4-oxadiazole, 78, for preparing high specific activity radioiodinated *m*-[¹²⁵I]iodobenzoylhydrazine, 79. The high specific activity radiolabeling was conducted at 100 °C for 1 h, followed by addition of trifluoroacetic acid/H₂O and heating for an additional 1.5 h. A 12% radiochemical yield of 79 was isolated from an HPLC.

Rea et al. have investigated the use of 2-hydroxy-5-[¹²⁵I]iodo-3-methylbenzoylhydrazine, 81, for protein labeling (277). The radioiodinated 81 was prepared (carrier-added) from an acylhydrazone synthon, 1,3,4-oxadiazole derivative 80 (278), which was readily prepared from commercially available 3-methylsalicylic acid. This is an interesting phenolic compound as only one radiohalogenated isomer can be formed from a halogenation reaction due to the methyl substituent. Conjugation of the iodobenzoyl hydrazide 81 at pH 5 for 6–8 h with periodate-oxidized monoclonal antibody resulted in a conjugate

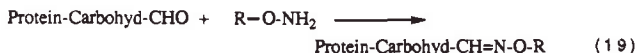
which was found to be unstable in vivo. Subsequently, reduction of the conjugated acylhydrazone with sodium cyanoborohydride provided a conjugate that was stable in vivo.

A radioiodinated acylhydrazone compound, 3-(3-[¹²⁵I]-iodo-4-hydroxyphenyl)propionyl carbohydrazide, 82a, has been prepared from the reaction of carbohydrazide with the Bolton–Hunter reagent, 3 (279). Good yields of the radioiodinated carbohydrazide were obtained. As with other phenolic reagents, two radioiodinated spots were observed by thin-layer chromatography, which the investigators attributed to the mono- and diiodo derivatives, 82a and 82b. The radioiodinated carbohydrazide was reacted with periodate-oxidized ribonucleosides (forming morpholine derivatives). Studies were also conducted with various other aldehydes and ketones to determine if this reagent could have a general application. The results suggest that it can; however, no studies of its application to oxidized glycoproteins were reported.

Studies were conducted at NeoRx to prepare *p*-[¹²⁵I]-iodobenzoylhydrazine, 84 (280). To prepare 84, the corresponding arylstannane, *p*-(tri-*n*-butylstannyl)benzoylhydrazine, 83, was synthesized from the reaction of hydrazine with methyl *p*-(tri-*n*-butylstannyl)benzoate. Radioiodination of 83 in CHCl₃ with carrier-added sodium [¹²⁵I]iodide resulted in a radiochemical yield of ca. 40% and a no-carrier-added yield of only 20%. Since the HPLC chromatograms indicated that only desired product and free iodide were present, higher amounts of the oxidant NCS were added. This resulted in production of unidentified polar products, with a decrease in overall radiochemical yields. An alternate path to the production of 84 was investigated. Very good radiochemical yields were obtained (>80%) when the *N*-hydroxysuccinimide ester 9b was reacted with a large excess of hydrazine. Separation of the excess hydrazine was accomplished on a reverse-phase C-18 Sep-Pak cartridge using MeOH as the solvent.

A preliminary investigation of the preparation of *p*-[¹⁸F]-fluorophenylhydrazine, 86, has been reported (281). The synthesis of 86 involved four steps: (1) preparation of *p*-[¹⁸F]fluoronitrobenzene by reaction of fluoride with dinitrobenzene, 85, (2) reduction of the nitro group to give *p*-[¹⁸F]fluoroaniline, (3) diazotization of the aniline, and (4) reduction of the diazonium salt with sodium cyanoborohydride. Phenylhydrazine 86 was generated in situ and reacted with keto compounds to form stable derivatives.

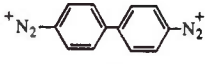
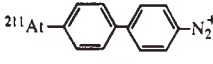
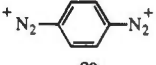
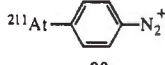
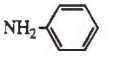
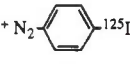
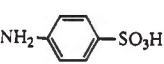
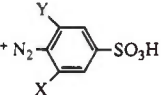
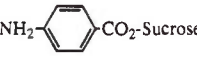
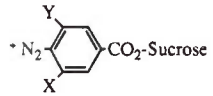
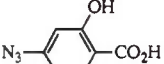
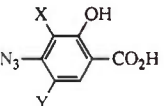
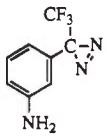
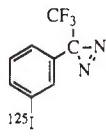
3. *Hydroxylamines*. A third aldehyde-reactive functionality is the hydroxylamine group. Reaction of hydroxylamines with aldehydes produce oximes as shown in eq 19, which are also stable functionalities (282).



No literature reports of radiohalogenation reagents which contain a hydroxylamine group were found. Preparation of such a reagent and its comparison in carbohydrate conjugations with amines, hydrazines, and acylhydrazines would be an important contribution to this area of study.

D. Conjugates That React Nonspecifically. Conjugation with specific chemical groups on proteins are most often sought; however, for some applications selectivity provided by reaction with a particular type of residue may not be important, or even desired. A listing of the reagents that I have classified as nonspecific conjugation reagents is given in Table VII. A description of some of the

Table VII. Radiohalogenation Reagents Which React in a Nonspecific Manner^a

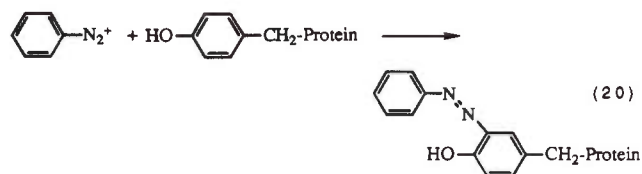
starting reagent	radiolabeled reagent (for protein conjugation)	% radiolab yield	% conjugn yield	ref
A. Diazonium Salts				
		NR	50 (overall)	130
		NR	23-57 (overall)	285
		NR	NR	286
	 94a: X = ¹²⁵ I, Y = H 94b: X = Y = ¹²⁵ I	95	>85	287
	 96a: X = ¹²⁵ I, Y = H 96b: X = Y = ¹²⁵ I	85-90	NR	288
B. Aryl Azides				
	 98a: X = ¹²⁵ I, Y = H 98b: X = H, Y = ¹²⁵ I 98c: X = Y = ¹²⁵ I	NR	7-16 (not all covalent)	292
C. Diazirines				
		60-80	5%	293

^a NR = not reported.

chemistry related to the reagents classified in this manner is given in the following text.

1. Diazonium Salts. Diazonium salts can undergo a number of different reactions with functional groups (283). For example, under basic conditions aryl diazonium salts will react with aromatic ring compounds to form bis-aryl compounds. Diazonium salts will also react with amines under basic conditions to form triazines. Under neutral or basic conditions, diazonium salts will undergo electrophilic reactions with phenols (phenoxide ions) to form diazo linkages.

It is believed that a primary reaction of aryl diazonium salts with proteins is reaction of tyrosine residues as depicted in eq 20. Reaction with tyrosine can produce



either the mono- or disubstituted derivatives as is observed

in electrophilic halogenations. Similarly, histidine imidazole rings can undergo electrophilic substitution reactions. Amines on other amino acids also react with aryl diazonium salts, but their reactions are thought to take place at a slower rate than those of tyrosine or imidazole (284).

The use of diazonium salts to incorporate astatine-211 into aromatic compounds, as an alternative approach to direct labeling of proteins, was reported in 1955 (130). Indeed, in that very early report it was shown that diazotized benzidine, 87, could be halogenated with ²¹¹At to form the astatinated diazo compound 88, which was subsequently reacted with bovine serum albumin (BSA) to form a stable conjugate. Similarly, Wunderlich et al. reported that diaminobenzene could be diazotized to form *p*-diazobenzene, 89, labeled with astatine to form the astatinated diazobenzene 90, and conjugated with rabbit immunoglobulin (285). The conjugation reaction was conducted at pH 8 and room temperature for 1 h and was quenched by addition of an excess of phenol.

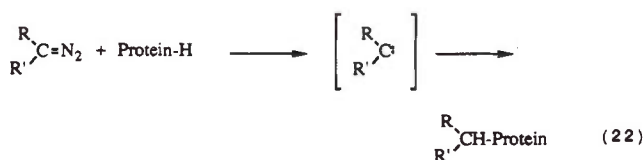
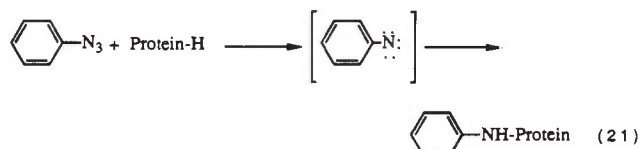
Hayes and Goldstein described the use of *p*-diazo[¹²⁵I]-iodobenzene, 92, for labeling proteins that contained

sulfhydryl groups which could not be modified without affecting the biological activity (e.g. enzymes) (286). Preparation of the radioiodinated diazonium salt **92** was accomplished through iodination of aniline, **91**, followed by diazotization. Conjugation with the protein was conducted at pH 9–10 for 1 h. The investigators indicated that a quantitative yield was obtained (Table 2 in that paper); however very few details on the results of labeling were given.

Labeling of proteins on red blood cell membranes with diazotized [¹³¹I]diiodosulfanilic acid, **94**, has been reported by Sears et al. (287). Radioiodinations of sulfanilic acid, **93**, gave the diiodosulfanilic acid, which was subsequently diazotized to form **94**. Conjugation of the radioiodinated diiodosulfanilic acid was carried out at 4 °C, pH 7.4. Raising the temperature in the conjugation to 24 or 37 °C gave lower conjugation yields, as did changing the pH to 7.0 or 7.8. The monoiodo derivative **94a** can be prepared from [¹²⁵I]iodosulfanilic acid, which is commercially available (NEN/Du Pont).

Another reagent that has been conjugated to proteins through reaction of a diazonium salt is *O*-(4-diazo-3,5-di-¹²⁵I)iodobenzoyl)sucrose, **96** (288). Preparation of **96** [structure not defined] was accomplished by radioiodination of (*p*-aminobenzoyl)sucrose, **95**, with (carrier) added NaI. The investigators reported that 1.7–1.8 equiv of iodine per molecule were obtained, indicating that a mixture of mono- and diiodo derivatives **96a** and **96b** may have been obtained. Conjugation was conducted at pH 8.1, 0 °C, for 2 h. It was postulated that the presence of sucrose on the radiolabeled compound would cause retention of the metabolized radioactivity in the organelles of the cells that metabolized it. Retention of activity was found to be largely in the mitochondrial and lysosomal fractions, and the investigators were able to determine the uptake of lactate dehydrogenase in the liver and spleen of rats using this reagent.

2. Photogenerated Species. Very fast, nonspecific reactions can be of value for coupling a radiohalogenated molecule with a protein. These types of reactions are used when attempting to detect interaction with "active sites" of proteins. They can also help provide information on such things as molecular structure of receptor proteins and transport proteins, membrane structure, protein-nucleic acid interactions, and antibody binding (289). The need to allow for an interaction prior to reaction makes the use of photolabile agents particularly attractive. There are two types of very reactive intermediates produced through photolysis of precursors that have been used extensively in protein labeling. These are aryl azides (290) and stabilized diazo compounds (i.e. acyl and phenyl substituted) (289). The photolysis of these reagents to produce aryl nitrenes and carbenes is shown in eq 21 and 22, respectively. Both of these reactive intermediates will



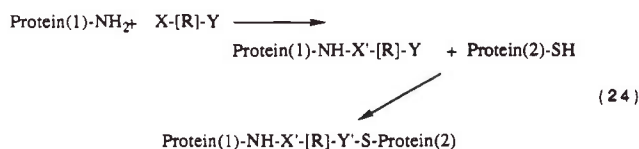
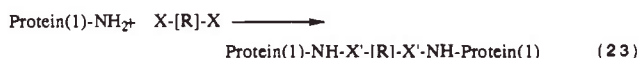
undergo nonspecific reactions (291) in the labeling of

proteins. For the purposes of this review, most of the applications of photolabeling reagents are included in the section on cross-linking of proteins.

An aryl azide radioiodinated photolabeling reagent (non-cross-linking) has recently been reported by Pandey et al. (292). The reagent, 4-azido-3-[¹²⁵I]iodo-2-hydroxybenzoic acid, **98**, was produced by radioiodination of 4-azidosalicylic acid, **97**. The investigators made no attempt to purify the radiolabeled **98** and did not determine whether it was the monoiodinated derivatives (**98a** and **98b**) or the diiodinated derivative (**98c**). They noted that two spots were observed by TLC which they believed could be the mono- and diiodo derivatives. As might have been expected for this type of reagent, low conjugation yields were obtained. Of the material associated with the protein after photolysis, a varying quantity (41–72%) was actually covalently attached to the protein.

A carbene-generating radioiodinated photolabeling reagent, 3-(trifluoromethyl)-3-(*m*-[¹²⁵I]iodophenyl)diazirine, **100**, has been reported for application to labeling in hydrophobic regions of membranes (293) and microsomal proteins (294). The structurally interesting diazirine group has been used for photolabeling (295, 296) and its chemistry has been reviewed (297). The diazirine **100** was produced from 3-(trifluoromethyl)-3-(*m*-aminophenyl)diazirine, **99**. Radioiodination of **99** was accomplished via two different synthetic routes. The initial studies incorporated the radioiodine in the first step of a multiple step synthesis and only 5% recovery of activity (as compound **100**) was obtained. A second route employed a diazonium salt decomposition for introducing the radioiodine in the final synthetic step. Very good radiochemical yields of the desired compound were obtained. Photolysis conducted at 0 °C under N₂ resulted in ca. 5% of the radioactivity being bound to protein.

E. Cross-Linking Reagents. Linking proteins to solid supports and other molecules (e.g. other proteins) has provided valuable tools to isolate, purify, stabilize, and elucidate biochemical interactions of the proteins. Discussion of the applications of cross-linking reagents is outside of the scope of this review, but can be found in a number of literature articles (170, 298–301). In essence there are two separate conjugation reactions occurring with the cross-linking reagents. Control of the two conjugation reactions when the reactive species are the same (homobifunctional) can be obtained by adjustments to the reaction conditions. More commonly the cross-linking compound will have two different reactive groups (heterobifunctional) for cross-linking of proteins. These two variations on cross-linking compounds are shown in eqs 23 and 24. Many of the cross-linking reagents contain a



photolyzable aryl azide group. Radiohalogenation (generally with ¹²⁵I) of the protein can be carried out separate from the cross-linking process by direct labeling (302, 303) of the protein or through the use of conjugates such as the Bolton-Hunter reagent (304), but radiohalogen labeling of the cross-linking reagent allows for monitoring of the first conjugation reaction and, subsequently, the cross-linking reaction.

Cross-linking of proteins with other materials (e.g. proteins, solid supports) can be accomplished with reagents that form stable attachments, but for some applications cross-linking reagents that can be cleaved following cross-linking are desired. These two types of radiohalogen labeled cross-linking reagents are discussed separately in the following text. A list of cross-linking reagents that contain radiohalogens is given in Table VIII.

1. Noncleavable. Three cross-linking reagents, *N*-succinimidyl 4-azidosalicylate, 101, *N*-succinimidyl *N*-(4'-azidosalicyl)-6-aminocaproate, 103, and *N*-succinimidyl *N*-(*N*-(4-azidobenzoyl)glycyl)tyrosinate, 105, were prepared and radioiodinated by Ji et al. (305, 306). Initial radioiodinations were conducted in acetone and it was reported that "iodine-125 incorporates quickly and efficiently" into 101 and 103 (one major radioactive spot by TLC), but radioiodination of 105 "produced several radioactive spots". In a subsequent paper by these investigators, the radioiodination of salicylate 101 was increased from 3% in acetone to 63% when other solvents "such as aqueous acetonitrile, dimethylformamide, or dimethyl sulfoxide" were used. The radioiodinated salicylate 102 (a-c?) was conjugated and photolyzed to cross-link the α and β chains of human choriongonadotropin.

More recently fluorinated aryl azide containing compounds have been used for protein cross-linking. The fluorinated aryl azides have been used because singlet nitrenes are generated from them upon photolysis, and the singlet nitrenes have been found to be efficient at inserting into a variety of bond types (307). Also, the fluorinated aryl azides may be less selective in their reactivity than nonfluorinated aryl azides as they do not produce the ring-expanded azepine intermediates which are (selectively) reactive with nucleophiles such as lysine amines (308, 309).

Crocker et al. have described the synthesis and iodination of *N*-succinimidyl *N*-(4-azido-2,3,5,6-tetrafluorobenzoyl)tyrosinate, 107 (310). Iodination of 107 with an excess of iodide yielded the diiodo derivative 108b ($X = Y = ^{127}\text{I}$) in 81% isolated, purified yield. The radioiodination of 107 was not reported. Photolysis studies demonstrated that very good cross-linking yields could be obtained (to 40%). Similarly, Keana et al. have described fluorinated aryl azide cross-linking reagents that could be radioiodinated (311, 312). These investigators synthesized *N*-succinimidyl 2-amino-4-azido-3,5,6-trifluorobenzoate, 109, and *N*-succinimidyl 3-(4-azido-2,3,5,6-tetrafluorobenzamido)-5-aminobenzoate, 111. They iodinated these two compounds to prepare 110 and 112 by in situ generation of the respective diazonium salts. In the reaction of 109 incomplete iodination was obtained using an excess of NaI at 0 °C for 30 min. Iodination of 111 gave the desired compound by addition of a large excess of KI at 5 °C for 40 min. Photolysis of 110 and 112 gave results that indicated photodeiodination had occurred with both compounds. Photodeiodination had also been observed for nonfluorinated aryl azides (313).

Another cross-linking reagent that is activated by photolysis is 3-(trifluoromethyl)-3-(*m*-isothiocyanophenyl)diazirine, 113, which was reported by Dolder et al. (314). Radioiodination to form 114 was found to be difficult with low yields being obtained. A major iodinated byproduct was obtained which the authors felt did not contain the isothiocyanate functionality. Attempts to radioiodinate the amino precursor to the isothiocyanate also gave poor yields and a number of byproducts. The amino group was found to be very sensitive to oxidation and some attempts to halogenate the amino compound

caused the loss of the amino group. Low cross-linking yields were also reported. The radioiodinated diazirine 114 is commercially available (Amersham).

A homobifunctional radiohalogenated cross-linking reagent has been developed by Lawton et al. (315-317). The reagents, termed equilibrium transfer alkylation cross-link reagents (ETAC reagents), are believed to generate sequential α,β -unsaturated ketones which are reactive with nucleophilic functional groups on proteins. A radioiodinated cross-linking reagent, *N*-[4-[2,2-bis[(*p*-tolylsulfonyl)methyl]acetyl]benzoyl]-4-[^{125}I]iodoaniline, 116, was prepared by reaction of [^{125}I]iodoaniline with the carboxylic acid chloride 115. This reaction gave very low yields of the radioiodinated 116. However, cross-linking using 116 was accomplished in high yields. The cross-linking yields were quite dependent on pH and on the degree of antibody reduction with 2-mercaptoethanol.

In our recent studies at the University of Washington, a stannylphenethylamine derivative, *N*-[4-[2,2-bis[(*p*-tolylsulfonyl)methyl]acetyl]benzoyl]-4-(tri-*n*-butylstannyl)phenethylamine, 117, has been prepared and radioiodinated to give 118 in high yields (318). This method of radioiodination of the ETAC molecules was chosen as we wanted to obtain better radiolabeling yields and higher specific activity labeling of ETAC cross-linking molecules. Cross-linking of antibody Fab' fragments to prepare modified F(ab')₂ has been achieved in good yield with this reagent.

2. Cleavable. For some applications it is desirable to be able to separate the cross-linked proteins after isolation. To accomplish this, some radioiodinated cross-linking reagents that can be cleaved in vitro have been developed.

A radioiodinated cross-linking reagent, *N*'-sulfosuccinimidyl *N*-[4-[(*p*-azido-*m*-[^{125}I]iodophenyl)azo]benzoyl]-3-aminopropionate, 120, has been reported by Denny and Blobel (319). Preparation of 120 was accomplished by radioiodination of *N*-[4-[(*p*-aminophenyl)azo]benzoyl]-3-propionic acid, 119, followed by diazotization/azide incorporation and esterification. The overall incorporation of radioiodine was low due to the multiple steps involved and the chromatographic separation of the radioiodinated aniline from 119, although a high specific activity was obtained. A 44% incorporation of the radiolabeled 120 into protein A attached to a Sepharose support was obtained. The derivatized protein A-Sepharose was then photolyzed in the presence of human serum. The investigators indicated that using the 366-nm light for photolysis did not release any free iodide. Once irradiated, the proteins were treated with sodium dithionite which had been shown to reduce the azo bond in similar nonradio-labeled reagents (320). Murphy and Harris have reported that improved radioiodination yields (10-14%) for 120 were obtained when it was reacted with aminodextran prior to radioiodination (321). The radioiodinated reagent 120 (*N*-hydroxysuccinimide ester) is commercially available as the Denny-Jaffe reagent (NEN/Du Pont).

Another cleavable cross-linking reagent, *N*-sulfosuccinimidyl 2-(*p*-azidosalicylamido)-1,3'-dithiopropionate, 121, has been studied (322). Wollenweber and Morrison reported coupling 121 to a *Escherichia coli* lipopolysaccharide (LPS) and subsequently radioiodinating it. A moderate radiolabeling yield was obtained and no evaluation of the possible isomeric products (122a-c) was discussed. Cleavage of the disulfide in 122 with DTT indicated that the radioiodine had been incorporated into the phenolic ring as expected. Cross-linking of 122 with serum albumin proteins (for which an affinity with LPS had been previously noted) with UV irradiation at 254 nm

Table VIII. Radiohalogenation Reagents That Cross-Link^a

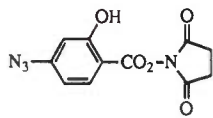
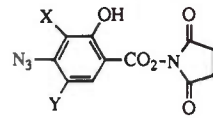
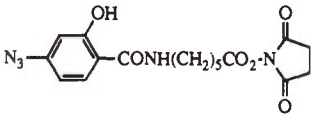
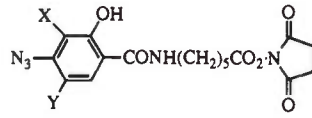
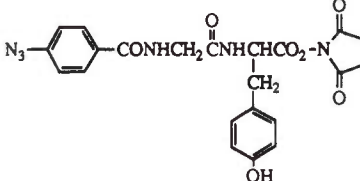
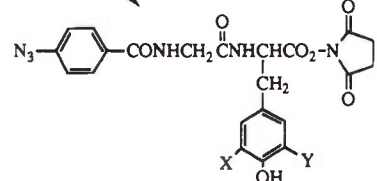
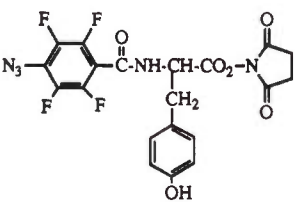
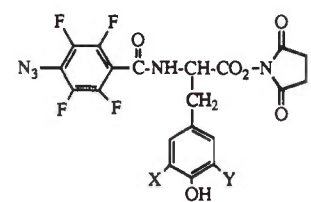
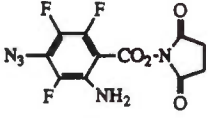
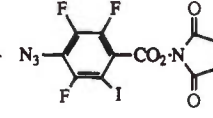
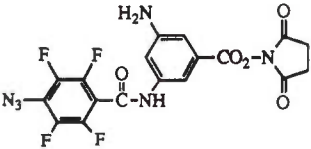
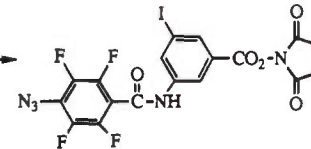
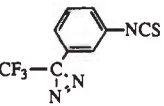
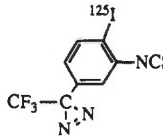
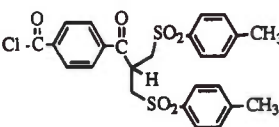
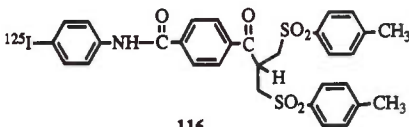
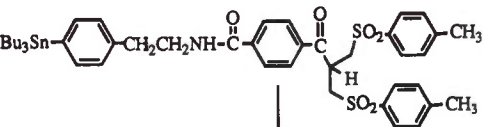
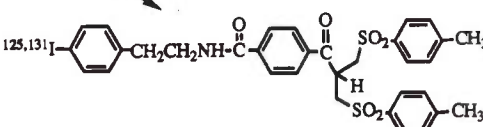
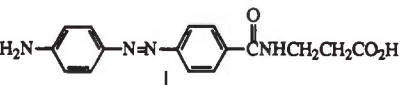
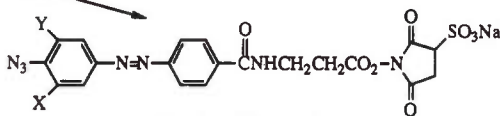
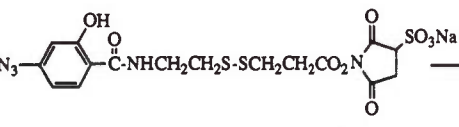
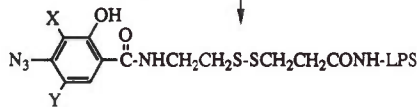
starting reagent	radiolabeled reagent (for protein cross-linking)	% radiolab yield	% cross-link yield	ref
A. Noncleavable Cross-Linking Reagents				
 101	 102a: X = ¹²⁵ I; Y = H 102b: X = H; Y = ¹²⁵ I 102c: X = Y = ¹²⁵ I	63	NR	306
 103	 104a: X = ¹²⁵ I; Y = H 104b: X = H; Y = ¹²⁵ I 104c: X = Y = ¹²⁵ I	NR	NR	305
 105	 106a: X = ¹²⁵ I; Y = H 106b: X = Y = ¹²⁵ I	NR	NR	305
 107	 108a: X = I; Y = H 108b: X = Y = I	NR	NR	310
 109	 110	NR	NR	311
 111	 112	NR	NR	311
 113	 114	<16	NR	314

Table VIII. (Continued)

starting reagent	radiolabeled reagent (for protein cross-linking)	% radiolab yield	% cross-link yield	ref
A. Noncleavable Cross-Linking Reagents (Continued)				
 115	 116	1	30–85	315
 117	 118	75–95	10–50	318
B. Cleavable Cross-Linking Reagents				
 119	 120a: X = ¹²⁵ I; Y = H 120b: X = Y = ¹²⁵ I	5	1	319
 121	 122a: X = ¹²⁵ I; Y = H 122b: X = H; Y = ¹²⁵ I 124c: X = Y = ¹²⁵ I	30	NR	322

^a NR = not reported.

gave "significant incorporation of ¹²⁵I into the serum albumins". The radioiodinatable cross-linking reagent 121 is commercially available (Pierce).

V. FUTURE DIRECTIONS

Radiohalogenated proteins have been instrumental in gaining knowledge about many basic concepts of protein biochemistry and have been used in medical applications such as radioimmunoassay. However, it seems like that radiohalogenated protein conjugates will become increasingly important in future biochemical studies and will be used in many more medical applications. Although the

majority of studies and applications of radiohalogenated proteins in the future will likely continue to use direct labeling with radioiodine, some of the studies will be best conducted with radiohalogenated conjugates. While commercially available radioiodination reagents such as the Bolton–Hunter reagent will continue to be used, newer conjugation reagents containing organometallic intermediates should become commercially available in the near future because of the advantages that they can provide over phenolic conjugates. These considerations aside, further studies will be conducted involving radiohalogenated conjugates, either those described in this review or

newly designed conjugates. In a majority of protein conjugate studies, design and development of a new conjugate will not be necessary as it should be possible to adapt one of the compounds listed in the tables in this review. However, it should be noted that many of the compounds listed in Tables IV–IX have undergone limited investigation and will not be readily adaptable to all labelings without further investigation and optimization of the radiohalogenation chemistry or conjugation chemistry, or both. This review has been purposefully broad in its scope so decisions can be made on which chemistry might improve labeling or conjugation yields when new reagents are designed.

A. Design of Conjugates. Many of the future studies of radiohalogenated proteins will be conducted in vivo. One very important aspect of conjugate labeling of proteins is that desirable chemical properties can be designed into them. For instance, radiohalogenated protein conjugates can potentially offer some control of in vivo metabolism and secondary distribution, whereas direct labeling does not. Additionally, there is often a need to know with certainty that the radioactivity observed in vivo evaluations of radiohalogenated proteins is indeed reflective of the distribution of that protein. This issue is one of in vivo stability, which can be addressed by designing conjugates that do not release the radiohalogen by the action of enzymes or nucleophiles found in the biological system. A brief discussion on what we have learned about in vivo stability is given in the next section.

Some control of the pharmacokinetics and in vivo distribution of labeled protein and its metabolites may also be possible through conjugate chemistry. While it is important to have control of in vivo stability, it may be desirable to have lower stability in certain tissues such as kidney and liver such that the radiation dose to these organs can be minimized. Design of conjugates which have chemical moieties that are selectively cleavable in tissues such as kidney and liver may provide a method of clearing those tissues faster than would otherwise be possible with stabilized conjugates. Another important function that may be designed into conjugates is a means of retention of the radiohalogen at a targeted tissue (e.g. tumor). A number of biochemical and/or chemical processes are available which could provide mechanisms (e.g. alkylation, metabolic trapping, etc.) by which retention or trapping of the radiohalogen at the target tissue could be obtained. Examples of radiohalogenated protein conjugates which were designed to be cleaved in vivo or be metabolically trapped are listed in Table IX. A discussion of the chemistry associated with the listed conjugates is given in the following text.

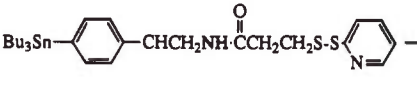
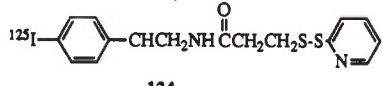
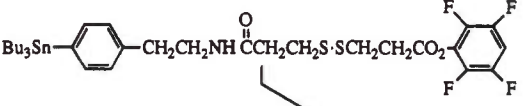
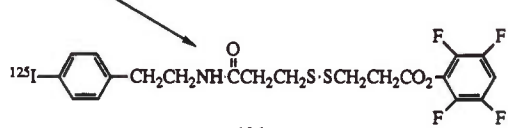
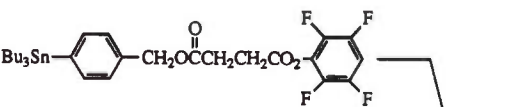
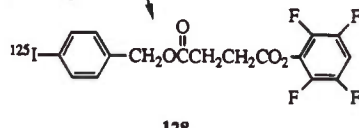
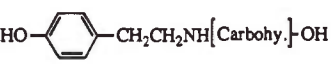
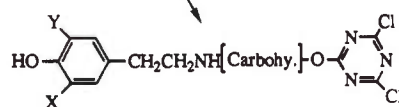
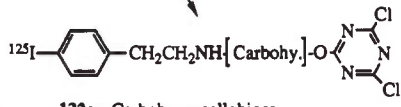
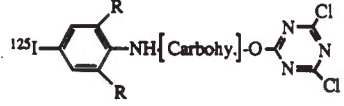
1. In Vivo Stability. A large amount of effort has been put into development of "stable" radiohalogenated conjugates because in vivo deiodination of directly labeled radioiodinated monoclonal antibodies, under investigation as imaging and therapeutic agents for cancer, has been considered a problem. Conjugates that contain phenolic rings have been shown to increase the in vivo stability relative to direct labeled counterparts, but some dehalogenation is still apparent. Importantly, it appears that the problem of in vivo dehalogenation has been solved by application of radiohalogenated nonactivated aromatic compounds such as the *p*-halobenzoate 10 and *m*-halobenzoate 12. However, it may be important to ask if the reason for the "stability" gained is really understood. Interpretation of what is occurring in the dehalogenation process is still not clear. That is, enzymatic dehalogenation (deiodination) has been perceived as a problem because

the presence of free radioiodide has been thought to be reflective of loss of radiolabel from the antibody, thus limiting the amount of activity that the antibody can deliver to the target tissue (e.g. cancer). However, the need to use "stable" conjugates may be dependent upon when the deiodination occurs. It may be that, as is widely held, deiodination occurs on the intact antibody (or fragment). On the other hand, deiodination may occur only after the protein has been metabolized to the individual amino acids and radioiodinated tyrosine is released. In this case, use of a stable radioiodination conjugation reagent may only provide a different excretion rate and secondary distribution of the radioiodine. The important point is that when a radiohalogenated protein or peptide is evaluated in vivo, metabolite studies must be conducted to help understand what is being observed when the tissues are counted. In vitro studies have shown that iodine can be lost from intact proteins (323), but questions of radiolysis of the label in those studies have been raised. Other investigators have interpreted observed differences in radioactivity of some tissues when radiometal- and radioiodine-label antibodies are coinjected as being due to rapid deiodination of antibodies (324, 325), but it may simply be due to the fact that the radiometal is retained in tissue after catabolism while the radioiodide freely leaves the tissue (326). Studies of the metabolites of *p*-[^{125,131}I]iodobenzoyl-conjugated antibodies have demonstrated that the primary metabolites are lysine adducts of the benzoate (and the *N*-acetyl derivatives) (327, 328). Catabolism to single amino acids in organs (329) and metabolites of protein conjugates containing lysine adducts have been reported previously (329, 330). Contrary to this, Zalutsky and co-workers have reported that free *m*-[¹³¹I]iodobenzoic acid and *m*-[²¹²At]astatobenzoic acid have been observed as the major metabolites of radiolabeled antibodies when *m*-halobenzoate is used as a conjugate (211). Other investigators have also found examples where the free carboxylic acid has been released from protein lysine amine conjugates (331). The difference in metabolites for the two radiohalogenated benzoyl labels may help explain the tissue differences observed in comparative studies (197, 208, 212). These results indicate that small variations in the structure of radiohalogenated protein conjugates could dramatically alter the fate of the metabolites from labeled proteins in vivo.

2. Cleavable Linkers. Applications to therapy using radiolabeled antibodies have been hindered in some studies by accumulation of radioactivity in the liver or kidney. A major improvement in the agent might be obtained if a selective release of the radionuclide in excretory tissues (e.g. liver and kidney) without (or with minimal) effect on tumor-associated radioactivity. This concept has prompted the development of conjugates that have chemical moieties, or linkers, which are cleavable in vivo. Studies of protein drug and toxin conjugates (269) have provided examples of release of drug through esters (332), acid-cleavable linkers (333, 334), disulfide linkers (335), and proteolysis of peptides (336).

Studies involving synthesis, radioiodination, and conjugation of radioiodinated disulfide containing compounds were conducted at NeoRx (337). Reaction of *p*-(tri-*n*-butylstannyl)phenethylamine with *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP, Pierce) provided disulfide-containing arylstannane 123. Radioiodination of 123 gave very high yields of the corresponding aryl iodide 124. Conjugation studies with radioiodinated 124 involving DTT-treated intact antibody gave highly variable yields. In vitro evaluation of an antibody conjugate of

Table IX. Radiohalogenation Reagents Containing Linker Functionalities

starting reagent	radiolabeled reagent (for protein conjugation)	% radiolab yield	% conjugn yield	ref
A. Metabolizable Linkers				
 123	 124	88-99	2-61	337
 125	 126	>90	25-50	337
 127	 128	>90	60	340
B. Nonmetabolizable Linkers				
 129a: Carbohy. = cellobiose	 130a: X = ¹²⁵ I, Y = H; Carbohy. = cellobiose 130b: X = Y = ¹²⁵ I; Carbohy. = cellobiose 130c: X = ¹²⁵ I, Y = H; Carbohy. = glucose 130d: X = Y = ¹²⁵ I; Carbohy. = glucose	80-95 83	46-70 55	344, 346 350, 351 350, 351
 131a: Carbohy. = cellobiose 131b: Carbohy. = glucose	 132a: Carbohy. = cellobiose 132b: Carbohy. = glucose	35 18	9-35 8-34	350, 351 350, 351
 133a: R = H; Carbohy. = cellobiose 133b: R = H; Carbohy. = glucose 133c: R = CH ₃ ; Carbohy. = cellobiose 133d: R = CH ₃ ; Carbohy. = glucose	 134a: R = H; Carbohy. = cellobiose 134b: R = H; Carbohy. = glucose 134c: R = CH ₃ ; Carbohy. = cellobiose 134d: R = CH ₃ ; Carbohy. = glucose	83 75 95 93	10-31 15-23 19-48 14-48	350, 351 350, 351 350, 351 350, 351
 135a: R = CH ₃ ; Carbohy. = cellobiose 135b: R = CH ₃ ; Carbohy. = glucose	 136a: R = CH ₃ ; Carbohy. = cellobiose 136b: R = CH ₃ ; Carbohy. = glucose	34 29	8-18 6-17	350, 351 350, 351

124 in PBS at 37 °C indicated that protein-bound radioactivity had only decreased to 82% by 6 days, and under the same conditions in serum it had decreased to 87%. In contrast to this, incubation of serum containing 2 and 4 mM cysteine decreased the protein bound activity to 18% and 9%, respectively, after 1.5 h at 37 °C. Problems with consistent conjugation yields led to a second approach to preparing disulfide conjugates.

A secondary approach to obtaining conjugates with disulfide bonds was to react *p*-(tri-*n*-butylstannyl)phenethylamine with another commercially available cross-linking compound, 3,3'-dithiobis(succinimidyl propionate) (DSP, Pierce). Initial attempts to produce the desired conjugate were fraught with difficulties as a mixture of desired compound, dimer containing two phenethylamines, and the compound where the *N*-hydroxysuccinimide ester was cleaved to the acid was obtained. Difficulties in purifying the desired compound from the mixture led to an alternate preparation. In the alternate preparation, an excess of DSP was used (to alleviate the dimer) and any *N*-hydroxysuccinimide ester remaining was hydrolyzed to the free acid prior to workup. Esterification of the free acid with DCC/tetrafluorophenol yielded the TFP ester of the DSP adduct of stannylphenethylamine, 125. Radioiodination of 125 was found to be facile with radiochemical yields consistently >90%. Conjugations with antibodies of radioiodinated 126 were conducted optimally at 37 °C for 30 min. The conjugate was purified by size-exclusion chromatography (Sephadex G-25; PD-10) to 94% with no high molecular weight aggregates being observed. In vitro evaluation of the radioiodinated DSP conjugate showed a minor drop in protein-associated activity at 37 °C in PBS for 2 h. Some aggregation or binding to serum proteins occurred in human serum over a 90-h period, but no loss of activity from proteins was noted. Treatment of an antibody conjugated to radioiodinated 126 with 10 mM DTT decreased the amount of protein-bound activity to 10% after 11 h, showing that the disulfide could be selectively cleaved. Unfortunately, the antibody conjugate of 126 was found to be quite unstable in vivo. Additional studies need to be conducted to determine if the in vivo stability could be increased by having hindered disulfides (338, 339).

Another cleavable linker that was studied at NeoRx was an ester-containing radioiodinated conjugate, tetrafluorophenyl *p*-[¹²⁵I]iodobenzyl succinate, 128 (340). Tetrafluorophenyl *p*-(tri-*n*-butylstannyl)benzyl succinate, 127, was prepared by condensation of *p*-(tri-*n*-butylstannyl)benzyl alcohol with succinic anhydride, followed by esterification with DCC/tetrafluorophenol. Radioiodination of the ester was accomplished in high yield. Conjugations with an intact antibody and an antibody Fab fragment were accomplished in good yields at pH 8.5, 37 °C for 30 min. Evaluations of the radioiodinated ester, 128, conjugated Fab in saline indicated that minimal (<10%), if any, hydrolysis of the activity from the protein occurred over 30 h. In contrast to that, nearly 25% of the radioactivity was released from the protein in serum over that same period. In vivo evaluation of the Fab ester conjugate indicated that at 4 h, when a large differential would be of optimal value, its percent dose/gram (%D/g) in kidney was nearly identical to that of the same Fab radioiodinated with *p*-iodobenzoate, 10c. However, at 20 h the kidney activity (%D/g) was only 26% that of the *p*-iodobenzoate-labeled Fab. These results might indicate that metabolism of the ester had occurred after metabolism of the protein. More rapid cleavage of the ester (i.e. from

the nonmetabolized Fab) might be attained if a diester was used as a linker (341–343).

3. Nonmetabolizable Linkers. In contrast to removal of activity from tissues, when delivering imaging or therapeutic radiohalogen nuclides to tumors it would be of great benefit to be able to trap (retain) the radionuclide at the tumor. Some studies in the literature have shown that it is possible to metabolically trap radioiodine (probably in lysosomes) by attaching a molecule containing the radioiodine (usually tyramine) to a carbohydrate moiety. Pittman et al. coupled tyramine to cellobiose by reductive alkylation with sodium cyanoborohydride to form the adduct 129a and radioiodinated the adduct (344). The radioiodinated tyramine–cellobiose was then coupled to cyanuric chloride (345) to form 130 (a,b?), which was immediately coupled to a protein by reaction at pH 7.0–7.5 for at least 1 h. Very good radiolabeling yields and protein conjugation yields were obtained using their procedures. High retention of activity (71%) was obtained after 24 h in the liver and bone marrow when asialotransferrin, a protein targeted for uptake in liver and bone marrow cells, was coupled with radioiodinated tyramine–cellobiose–cyanuric chloride and injected into rabbits.

Strobel et al. employed the same labeling method as Pittman et al. for radioiodination/conjugation of lactitol, cellobitol, dilactitol, and dicellobitol to a model carrier protein asialofetuin (ASF) (346). The results obtained indicated that radioiodinated glycoconjugates of ASF increased the liver retention, where ASF is metabolized, by a factor of 5 in 24 h. Disubstituted [¹²⁵I]tyramine glycoconjugates were retained more efficiently than the smaller monosubstituted conjugates. More recently, Maxwell et al. have described the use of much larger radioiodinated tyramine–inulin protein conjugates to slow the release of radiolabel (through catabolism) and improve the retention (347).

Ali et al. have used radioiodinated tyramine–cellobiose to radiolabel monoclonal antibodies in an attempt to increase the residence time of the radioactivity in a tumor model (348). These investigators found that good labeling and conjugation yields could be obtained while the immunoreactivity of the antibody was retained. They also found that tumor uptake and retention were greater with the radioiodinated tyramine–cellobiose conjugate than with directly labeled antibody, while other tissues were very similar. Welch et al. also investigated tyramine–cellobiose, 130, labeled antibodies (349). In contrast to the Ali et al. results, they found that the amount of antibody in the tumor was not increased but was retained longer. They also observed an increase in the liver activity over direct labeling. More recently, Ali et al. have reported the preparation, radioiodination, and conjugation of ten carbohydrate adducts (129a,b, 131a,b, 133a–d, 135a,b) (350, 351). In biodistribution studies, they found prolonged retention of the tyramine–cellobiose-labeled antibody in tumor over a direct-labeled control and again found normal tissues to be similar to the direct-labeled control.

B. Direct Labeling of Conjugates. It is very likely that new radiohalogenated conjugates will be developed which will have properties that are designed for specific applications; however, application of conjugate labeling methods may not be widely used even then, as the chemistry can be fairly difficult to conduct. Therefore, the challenge for the chemists will be to develop simple methods for radiolabeling proteins after the small molecule is conjugated. To do this, methods of producing rapid, high specific activity, high-yielding radiohalogenations of

proteins under mild conditions must be developed. This will be more challenging for some radionuclides than others, but all radionuclides will require developing compounds that are more reactive than any of the other reactive functional groups on the protein. Very reactive functional groups on proteins such as tyrosines (in electrophilic radiohalogenations) may have to be deactivated by reversible modification prior to the radiohalogenation reaction.

VI. RADIATION SAFETY CONSIDERATIONS

A detailed description of radiation safety procedures and shielding requirements when conducting experiments with radiohalogens is beyond the scope of this review. (This information can generally be obtained from radiation safety personnel at your institution.) However, the volatile nature of many radiohalogen species (e.g. dihalogens, acid halides) makes handling of these materials somewhat different than other radioactive materials. This is particularly true for handling radioiodine, as it is sequestered by the thyroid gland. Thus, a few statements about precautions that may be taken when conducting radiohalogenation reactions are given below.

The volatile nature of radiohalogen dictates that radiohalogenations always be conducted in a fume hood, vented glovebox, or (if large amounts of activity are used) in a "hot cell". For radioiodinations, a Plexiglas "iodination box" which has its own exhaust fan and charcoal filter is available commercially (Radiation Physics, Beltsville, MD). We have placed an iodination box within a fume hood and use it for all routine labelings. The charcoal filter in the unit is easily accessible and can be readily changed. Astatine-211 labelings are conducted in a glovebox which is vented through a charcoal filter into the iodination chamber, where its exhaust is passed through a second charcoal filter. Radiohalogenation reactions are routinely conducted in reaction vessels which are vented to the inside of the iodination box or glovebox through a (10-mL) syringe filled with charcoal. The effluent of the fume hood used for radioiodinations (other halogens?) should be monitored for release of radioactivity. While it may not be your task, an inexpensive method of monitoring for release of radiohalogens in the fume hood exhaust has been described by safety personnel at the University of Wisconsin which may be adapted to your facility (352).

As with handling any radioactive material, the appropriate laboratory attire must be worn. We routinely double glove (two gloves per hand) as a safety precaution against volatile halogens penetrating the first glove layer. We also use commercially available "sleeve protectors" to aid in decreasing the contamination of the lab coat sleeves when using the iodination box. Monitoring with appropriate equipment is conducted continually throughout an experiment. Between 24 and 72 h post radioiodinations, investigators should have a thyroid bioassay conducted. This is usually done by radiation safety personnel.

We have found that the radiation waste poses a significant health risk if it is not treated properly. This is particularly true of radioactivity that is left behind in glassware and on chromatography columns. Therefore, we routinely elute base (e.g. 0.1 N NaOH) over chromatography columns used in radiohalogenation reactions, rinse the reaction vessels and other glassware used in the labeling with base, and add base to any liquid waste from the radiohalogenations prior to storage or disposal. Once rinsed with base, the items are placed in a Zip-loc bag which contains charcoal to adsorb any volatile halogen.

Handling of astatine-211 is minimized, and all labeling glassware and equipment remain in the glove box for a minimum of 4 d (13 half-lives) before cleaning for the next experiment.

Radiohalogenations at high levels of activity can present a radiation risk. However, the risk is minimal if the protein labelings can be done remotely. In radioiodinations with **9b**, we conducted high-level radioiodination reactions involving up to 1 Ci of activity. In those experiments we found that all of the mixing and moving of radioactivity could be accomplished at a distance (remotely) from shielded sources. This was accomplished by using air pressure from syringes to move the activity from the reaction vessel into the protein solution and onto a purification column via sterile transfer tubes (cannulas). Indeed, several investigators have described similar remote labeling setups which could be adapted to almost any protein labeling experiment (353-355).

VII. SUMMARY

Direct labeling of proteins with radionuclides of iodine will continue to be the method of choice to answer questions addressed in many future studies. However, it seems likely that a increasing number of applications of radiohalogenated proteins will require, or benefit from, conjugate labeling. While many radiohalogen conjugates have been studied, a large proportion of them have only undergone preliminary studies to date, leaving a question of their overall utility. Phenolic conjugates give good radioiodination labeling yields, but mixtures of radiohalogenated products and problems with *in vivo* stability can be expected. This fact, along with the fact that phenolic compounds do not have a general application to radiohalogens, makes them less attractive than other alternatives.

Radiohalogen labeling through the use of organometallic intermediates has proven to be facile, resulting in high yields of high specific activity labeled small-molecule conjugates. Although the choice of which organometallic intermediate to use may depend somewhat on the radionuclide employed, arylstannanes appear to have the most general applicability. Fluorine-18 labeling of small-molecule conjugates has been best accomplished by *ipso* aromatic nucleophilic substitution (exchange) reactions. Radiohalogenated small molecules have been prepared which can be conjugated with specific functional groups (e.g. amines, sulfhydryl groups, and carbohydrates) or conjugated nonspecifically with groups in the proximity of the conjugate when it is photolyzed. On the basis of previous studies, good conjugation yields (i.e. 60-90%) can be expected for reactions with specific groups, whereas low yields (i.e. 1-5%) can be expected for conjugations with reactive nitrenes and carbenes. However, recent developments in the chemistry of conjugates that produce nitrenes and carbenes will likely improve the radiolabeling yields.

There have been too few comparative studies to readily assess which is the best approach to take when beginning a study involving radiohalogenation of a protein or peptide. However, it is clear that radiohalogenated conjugates of proteins can offer an advantage over direct labeling in that conjugates may be designed which provide some control over *in vivo* stability and secondary distribution of metabolites. Conjugates can be prepared which are designed to utilize *in vivo* biochemical processes to release a radiohalogenated small molecule from a tissue (i.e. kidney or liver) or retain the radioactivity at the target tissue (e.g. tumor). Aside from the designing of conjugates with

linking molecules for desired biological effects, the ultimate future goal for the radiolabeling chemist should be to prepare protein conjugates which can be radiohalogenated in a simple one-step procedure.

VIII. ACKNOWLEDGMENT

I would like to acknowledge all of the scientists that have contributed to the understanding of radiohalogenation reactions and development of radiohalogenated protein conjugates over the past 8 years. These include: Scott Rogers, Zita Svitra, Dr. Kent Anderson, and Wendy Stone for their assistance in organic synthesis and radiohalogen labeling at Los Alamos; Dr. Mark Hylarides, Dr. Stephen Hadley, Dr. David Jones, Joan Schroeder, Don Axworthy, Leah Grant, and Jerry Seubert for their assistance in organic synthesis and radiolabeling at NeoRx Corp.; Dr. Mary Ann Gray, Dr. Paul Beaumier, Karen Poole, Denise Dupont, and Joanne Stevens for their assistance in the animal studies conducted at NeoRx Corp.; Dr. Bob Vessella, James Stray, Dena Goffe, and Don Hamlin for their assistance in continuing studies at the University of Washington. I would also like to thank Dr. Robert Atcher, John Hines, and Ed Kemerite for their assistance in providing astatine-211 for our studies. I thank the following organizations for their support of radiohalogenated antibody research: DOE for support at Los Alamos National Laboratory and support in collaboration with Argonne National Laboratory; NIH for support under Grants N43-CM-57759, N43-CM-67759, and N43-CM-67939; NeoRx Corp. for their financial support; and University of Washington School of Medicine (BRSG 62-3126).

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