Fluorine-18 Labeling of Proteins

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Two fluorine-18-labeled reagents, methyl 3-[^{18}F]fluoro-5-nitrobenzimidate and 4-[^{18}F]fluorophenacyl bromide, have been prepared for covalent attachment of fluorine-18 to proteins. Both reagents can be prepared in moderate yields (30–50%, EOB) in synthesis times of 50–70 min. Reaction of these reagents with proteins (human serum albumin, human fibrinogen, and human immunoglobulin A) is pH independent, protein concentration dependent, and takes 5–60 min at mild pH (8.0) and temperature (25–37°C), in yields up to 95% (corrected). The ^{18}F-labeled proteins are purified by size exclusion chromatography.

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Proteins have been successfully radiolabeled with halogen radioisotopes [bromine-77 (*1,2*), iodine-131, iodine-125 (*3,4*)], metal radioisotopes [technetium-99 (*5*), indium-111 (*6,7*), gallium-68 (*8*)] and carbon-11 (*9,10*) (carbon-11-methylation). Interest in radiolabeled proteins remains high, with uses ranging from blood volume markers iodine-125 human serum albumin to tumor detection and therapy (radiolabeled monoclonal antibodies) (*11*).

Although fluorine-18 (^{18}F) is an attractive radionuclide due to its ease of production (reactor or accelerator), half-life (110 min), and mode of decay (positron emission), very little effort has been expended in the labeling of proteins with this radionuclide. In a single published report Muller-Platz et al. (*12*) labeled urokinase by way of the carbodiimide-mediated acylation of the protein with [^{18}F]fluoroacetic acid. As it is relatively simple to label proteins with generator-produced gallium-68 (^{68}Ga) (beta-plus emission, $t_{½}$ = 68 min), the need to develop labeling with ^{18}F might be questioned. There appears to be several advantages of ^{18}F over ^{68}Ga. The longer half-life (110 versus 68 min) may be important for studies of relatively slow biologic processes. As the positron emitted from ^{18}F is of lower energy than from ^{68}Ga, it is possible to administer larger amounts of ^{18}F (with the same absorbed radiation dose) despite the significantly longer half-life. Assuming only organ self-irradiation, MIRD calculations show lower absorbed doses for ^{18}F (*13*); a comparison of ^{18}F and ^{68}Ga absorbed doses for a selection of organs and whole body is shown in Table 1. As 50% more ^{18}F can be administered with the same critical organ radiation dose, and with the longer half-life, more counts will be available for imaging studies at extended time periods. For example, in studies of indium-111-labeled antibodies, it has become apparent that significantly more radioactivity is trapped in the liver as compared to radioiodine-labeled antibodies (*14*). This radioactivity is retained in the liver over a long time period (several days). If fluorination resembles iodination, then there will be less accumulation of ^{18}F in the liver, compared to the ^{68}Ga antibody, thus resulting in a lower absorbed dose to the liver.

As part of our continued interests in radiolabeled proteins and the preparation of positron-emitting radiopharmaceuticals, we describe here the synthesis of two ^{18}F-labeled reagents for protein labeling methyl 3-[^{18}F]fluoro-5-nitrobenzimidate and 4-[^{18}F]fluorophenacyl bromide, and their application to the radiolabeling of three model proteins; human serum albumin, fibrinogen, and immunoglobulin A (IgA).

MATERIALS AND METHODS

Tetrabutylammonium fluoride, tetrabutylammonium hydroxide, 3,5-dinitrobenzonitrile, acetonitrile, dimethylsulfoxide, sodium methoxide, 4-nitrobenzonitrile, 4-fluorobenzonitrile, 4-nitroacetophenone, 4-fluoroacetophenone, copper(II) bromide, and methyllithium were obtained commercially* as were human serum albumin, fibrinogen, and IgA.† Purified human fibrinogen (95–97% clottable) was a gift from Dr. Larry Sherman of the Missouri Red Cross Laboratories. SEP-PAKs‡ and iodine-125 human serum albumin were obtained commercially.§

Authentic samples of 4-fluorophenacyl bromide and 4-

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TABLE 1
Ratio of Radiation Dose to Various Organs from Administration of Equal Amounts (mCi) of Fluorine-18 and Gallium-68

	Organ				
Dose	Lung	Spleen	Kidneys	Liver	Whole body
$\frac{\text{dose } ^{18}F}{\text{dose } ^{68}Ga}$	0.59	0.63	0.63	0.69	0.88

nitrophenacyl bromide were prepared by reaction of the corresponding acetophenones with cupric bromide according to the procedure of King and Ostrum (*15*).

Mass spectra were obtained on a Finnigan 3200 spectrometer (70 eV electron impact, direct probe). Proton and ^{19}F NMR spectra were obtained on a Varian XL-300 spectrometer; proton chemical shifts are reported relative to internal tetramethylsilane standard, and ^{19}F chemical shifts relative to CCl_3F (trifluoroacetic acid internal standard).

High pressure liquid chromatography (HPLC) was done using a Spectra-Physics 8700 chromatograph, and one of the following systems: System A, Spectra Physics Spherisorb 10 ODS, 0.46 × 25 cm, 1/1 v/v 0.03*M* KH_2PO_4/CH_3CN; System B, Alltech silica gel, 0.45 × 25 cm, 80/20 v/v CH_2Cl_2/hexane; System C, Alltech silica gel, 1 × 25 cm, 80/20 CH_2Cl_2/hexane; System D, Alltech Shodex Protein WS 802.5, 0.8 × 25 cm, 50 m*M* phosphate buffer with 0.1*M* NaCl, pH 7.0–7.1; System E, Alltech Synchropax AX-300, 0.41 × 25 cm, solvent A 0.02M Tris acetate pH 8, solvent B 0.02*M* Tris acetate plus 0.5*M* sodium acetate, pH 8.0, 20 min linear gradient; System F, Waters μBondagel, 0.45 × 35 cm, 0.02*M* KH_2PO_4, pH 7.0. The chromatograph was fitted with a variable wavelength uv (254 nm for systems A, B, and C, 280 nm for systems D, E, and F) and a NaI(Tl) radioactivity flow detector. Retention times for intermediate and final products (determined using authentic standards) are shown in Table 2.

3-Fluoro-5-nitrobenzonitrile. To 0.5 ml of 1*M* tetrabutylammonium fluoride in THF was added 0.1 ml CH_3CN, and the solution evaporated to dryness. The residue was dissolved in 0.4 ml of DMSO and added to 500 mg of 3,5-dinitrobenzonitrile. The solution was heated at 135°C for 1 hr, cooled, and transferred to a short silica gel column. The silica gel was eluted with diethyl ether, and the organic washings combined and evaporated. Portions of the crude product were purified by semi-prep HPLC (System C R_T = 13 min). Yield of 3-fluoro-5-nitrobenzonitrile is ~42% (remainder 3,5 dinitrobenzonitrile). The isolated product was recrystallized from hexane-methylene chloride: m.p. 47–48°C; ^1H NMR ($CDCl_3$) δ 7.73 (m, 1H), 8.21 (m, 1H), and 8.24 (s, 1H); ^{19}F NMR ($CDCl_3$) Φ −104.5(t); mass spec m/e (rel int) 166 (M^+, 42), 120 (100).

Methyl 3-fluoro-5-nitrobenzimidate. To 5 mg of 3-fluoro-5-nitrobenzonitrile in 0.1 ml of methanol was added a solution of 10 mg sodium methoxide in 1 ml methanol. After 1 hr the reaction was quenched by addition of 1 ml of 2% Na_2CO_3 and the aqueous mixture extracted with diethyl ether. The ether was passed through two silica gel SEP-PAKs and evaporated to yield the crude benzimidate [90% yield, 95% purity by HPLC (System A); remainder unreacted nitrile]. This was recrystallized from hexane/CH_2Cl_2 and hexane/petroleum ether: m.p. 57–58°C; ^1H NMR ($CDCl_3$) δ 3.92 and 4.05 (two singlets, ratio 2:1, 3H, OCH_3), 7.68 and 8.28 (1H, NH), 8.88 (d, 1H), 9.15 (q, 1H), and 9.21 (d, 1H); ^{19}F NMR ($CDCl_3$) Φ −107.5 and 108.9 (two triplets, ratio 2:1) mass spec m/e (rel int) 168 (27), 167 (45, M+ − OCH_3), 121 (99, M^+ − OCH_3 and NO_2).

Production of [^{18}F]fluoride ion. The aqueous [^{18}F]fluoride ion was obtained using the $^{18}O(p, n)^{18}F$ nuclear reaction and a small volume $H_2{}^{18}O$ target, as previously described (*16*).

Methyl 3-[^{18}F]fluoro-5-nitrobenzimidate (3). A portion of the $H_2{}^{18}O$ target water (10–200 μl) was combined with 5 μmol of tetrabytylammonium hydroxide, 100 μl CH_3CN added, and evaporated (N_2 flow) in a platinum crucible. The residue was dissolved in 0.25 ml of dimethylsulfoxide and added to 1 mg of 3,5-dinitrobenzonitrile. The vessel was capped and heated (133°C) for 35 min, then cooled and 2 to 3 ml of dry diethyl ether added. The ether solution was passed through a silica gel SEP-PAK and evaporated. To the residue was added 0.3 ml of a 10 mg/ml solution of $NaOCH_3$ in methanol. After 5–7 min, the reaction was stopped by addition of 1 ml of 2% Na_2CO_3, and the aqueous mixture extracted with ether. The

TABLE 2
HPLC Systems

	Retention times (flow rates*)					
Product	A Anal. C_{18}	B Anal. silica	C Prep silica	D Shodex	E Synchropax	F Bondagel
2	7.5	6.8	13 (2)			
3	8.5	—	—	17 (2.5)	3 (1.5)	10
4	5.0	4.7	10.5	—	4 (1.0)	—
5	5.9	6.5	16.2	—	3	—
6	8.0	—	9.7	19	6.5	14
7	12.2	—	—	—	—	—
^{18}F-HSA	—	—	—	8.0	20 (1.5)	3.0
^{18}F-Fib	—	—	—	4.0 (2)	—	4.0
^{18}F-IgA	—	—	—	4.0 (2.5)	13.0 (1.2)	—

* Flow rates are 1.0 ml/min unless otherwise noted.

ether was passed through a silica gel or Florisil SEP-PAK and rotary evaporated. The residue was suitable for use in protein labeling experiments.

Typical radiochemical yields are as follows (all decay corrected): resolubilization 72–75%, 3-[^{18}F]fluoro-5-nitrobenzonitrile 42–45%, and methyl 3-[^{18}F]fluoro-5-nitrobenzimidate 85–99%.

4-[^{18}F]Fluorobenzonitrile (4). Numerous syntheses of this compound have been previously reported (*17–20*). In this work, 1–100 mCi of ^{18}F-fluoride ion (1–100 μl of $H_2{}^{18}O$) was combined with 2 μmol of TBA·OH and evaporated in a pyrex vessel. A solution of 4-nitrobenzonitrile (2 mg) in DMSO (100 μl) was added, and the solution heated (158°C) for 20–30 min. The product was isolated by C_{18} SEP-PAK chromatography and obtained as a dried (Na_2SO_4) solution in 2–2.5 ml of pentane. Yields were 70–80% (corrected). Radiochemical purity (HPLC) was 95–99% (Systems A and B). The product can be purified by prep. silica gel HPLC (System C). In all cases the crude product was used without purification.

4-[^{18}F]Fluoroacetophenone (5). To a solution of **4** (0.5–60 mCi) in pentane (2 ml) were added 500 μl of MeLi solution (1.0*M* in diethyl ether). After 3 min 5 drops of methanol were added, and the pentane boiled off. Methanol (0.5 ml) and conc. HCl (10 drops) were added, and the solution boiled for 5 min. The mixture was diluted with 10 ml of water and passed through a C_{18} SEP-PAK. The products were eluted with 2 ml of 1:1 $CHCl_3$/ethyl acetate, and the organic solution dried (Na_2SO_4). Yields of crude product were 50–76% (corrected, synthesis time 15 min). Radiochemical purity (Systems A, B, C) was 95–99% with the balance unreacted nitrile **4**. The product can be purified by prep. silica (System C) but in all cases was used without purification.

4-[^{18}F]Fluorophenacyl bromide (6: α-bromo-4-[^{18}F]fluoroacetophenone). To a solution of crude ketone **5** (0.2–35 mCi) in 2 ml of 1:1 $CHCl_3$/ethyl acetate were added 50 mg of cupric bromide and the mixture refluxed for 30 min. The mixture was cooled, diluted with 5 ml of diethyl ether, and passed sequentially through Florisil and silica SEP-PAKS. Yields are 85–90% (corrected). HPLC analysis showed up to 4 possible products: nitrile **4** and unreacted ketone **5**, monobromination produce **6** and dibromination product **7**. The ether solution of crude **6** was rotary evaporated, and the residue dissolved in 1 ml of ether. This ether solution was transferred to a small glass tube and evaporated with a slow flow of argon. This dried residue was used in the protein labeling experiments.

General procedure for protein labeling. The proteins were dissolved in 1 ml of borate buffer (0.033*M*, 0.1μ, prepared with $Na_2B_4O_7$ and pH adjusted with 2N HCl) to give protein concentrations of 0.1–10%, and 0.1–0.5 ml of these solutions added to the dried residue of ^{18}F-labeled reagent **3** or **6** in a small test tube. The samples were incubated (water baths) for desired times. Aliquots were then removed and injected onto the HPLC, using either system D, E, or F for analyses.

Purification of ^{18}F-labeled proteins. The ^{18}F-labeled proteins were isolated using 0.9 × 5 cm Sephadex columns (G-10 for [^{18}F]HSA, G-25 for [^{18}F]fibrinogen and [^{18}F]IgA) which were eluted with 0.9% saline solution. Small (0.5 ml) fractions were collected and counted. Identification of the ^{18}F-protein peak was made by injection of an aliquot onto one of the protein HPLC columns (Systems D, E, or F). Identification of unreacted starting reagents and hydrolysis products was made by injection onto the analytical C_{18} column.

In vivo studies with [^{18}F]HSA. Human serum albumin labeled with [^{18}F]fluorobenzimidate **3** or 4-[^{18}F]fluorophenacyl bromide **6** and purified by Sephadex-G-10 column was injected intravenously into three Sprague-Dawley rats (5–15 μCi per rat). Immediately after, [^{125}I]HSA (5 μCi) was injected into each rat. At designated times thereafter, blood samples were collected by intracardiac puncture, weighed, and counted for ^{18}F and ^{125}I.

RESULTS

Synthesis of methyl 3-[^{18}F]fluoro-5-nitrobenzimidate. The preparation of the ^{18}F-labeled methylbenzimidate is shown in Figure 1. Nucleophilic aromatic substitution of no-carrier-added [^{18}F]fluoride ion for nitro in 3,5-dinitrobenzonitrile yielded 3-[^{18}F]fluoro-5-nitrobenzonitrile in 42–45% isolated yields (corrected) and 95% radiochemical purity. The product was separated from unreacted [^{18}F]fluoride by simple filtration through a silica gel SEP-PAK. Without further purification, the nitrile **2** was allowed to react with excess sodium methoxide in anhydrous methanol to give excellent (85–99%) yields of the benzimidate **3**. Excess sodium methoxide could be removed using a silica gel or Florisil SEP-PAK, and the product was suitable for use in the protein labeling step (after evaporation of solvent). Radiochemical purity (HPLC, system A) was routinely 90%, with the remainder unreacted nitrile **2**. Overall yield was 20–33% (17–22% EOS), in a synthesis time of 45–50 min. For example, from 21.5 mCi of [^{18}F]fluoride were prepared 3.53 mCi of methyl 3-[^{18}F]fluoro-5-nitrobenzimidate.

The identities of the ^{18}F-labeled products were checked routinely by HPLC using co-injections of authentic standards. A sample of 3-fluoro-5-nitrobenzonitrile was prepared by fluoride for nitro substitution of 3,5-dinitrobenzonitrile, and the product separated from unreacted dinitro nitrile **1** by semi-prep HPLC. The samples of methyl 3,5-dinitrobenzimidate and methyl 3-fluoro-nitrobenzimidate were prepared by re-

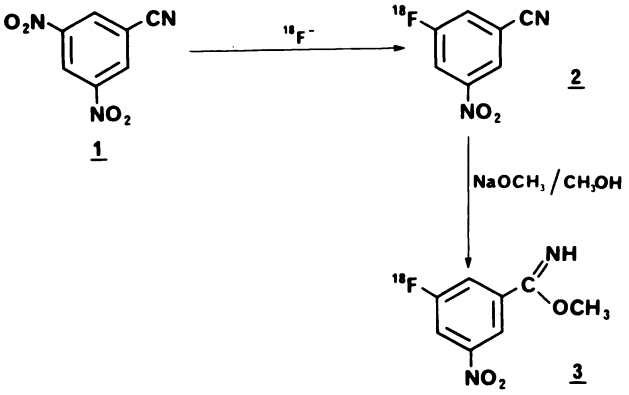

FIGURE 1
Synthesis scheme for methyl 3-[^{18}F]fluoro-5-nitrobenzimidate.

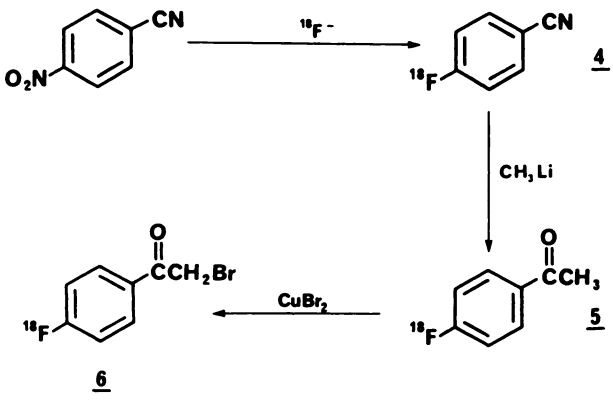

FIGURE 2
Synthesis scheme for 4-[^{18}F]fluorophenacylbromide.

action of the corresponding nitrile with sodium methoxide in methanol, by a modification of the procedures of Schaefer and Peters (*21*).

Synthesis of 4-[^{18}F]fluorophenacyl bromide. The preparation of this ^{18}F-labeled α-bromo ketone is shown in Figure 2, and involves three chemical reactions. 4-[^{18}F]Fluorobenzonitrile was prepared in good yields (70–80%) by aromatic nucleophilic substitution of no-carrier-added [^{18}F]fluoride ion for the nitro group of 4-nitrobenzonitrile. The product was isolated by simple C_{18} SEP-PAK chromatography. Reaction of the nitrile with excess methyllithium, followed by acidic hydrolysis, gave 4-[^{18}F]fluoroacetophenone in 50–70% yields (isolation by C_{18} SEP-PAK). HPLC analysis of the crude product showed a radiochemical purity of 95–99%, with 1–5% unreacted nitrile. Bromination of the methyl ketone was performed with cupric bromide in refluxing chloroform—methyl acetate, to yield a mixture of desired α-bromo ketone (85–90%), unreacted ketone (5–7%), and the α,α-dibromination product (5–7%). Yield of crude product was excellent (85–90%, corrected for decay) with the product purified using silica and Florisil SEP-PAKS.

Overall isolated yields of 4-[^{18}F]fluorophenacyl bromide (85–90% purity) were from 28–40% (decay corrected) in a synthesis time of 75 min. For example, from 55 mCi of [^{18}F]fluoride could be prepared 10.1 mCi (29%) of 4-[^{18}F]fluorophenacyl bromide.

Stability of methyl 3-[^{18}F]fluorobenzimidate. The stability of the [^{18}F]fluorobenzimidate in aqueous buffer was determined. In 0.033*M* borate buffer, pH 8.0, the imidate **3** undergoes <10% hydrolysis over a two-hour period (Table 3). Analyses were done by HPLC (System A), by timed injection of aliquots with identical volumes.

TABLE 3
Stability of Methyl 3-[^{18}F]Fluoro-5-Nitrobenzimidate in 0.33 *M* Borate Buffer, pH 8.0, 25°C

Time (min)	% Imidate	% Change
0	87.7	0
5	87.5	0.2
20	86.9	0.9
40	85.7	2.3
100	81.2	7.4

TABLE 4
pH Dependence of HSA Labeling with Methyl 3-[^{18}F] Fluoro-5-Nitrobenzimidate (**3**) and 4-[^{18}F]Fluorophenacyl Bromide (**6**). Conditions: 0.033M Borate Buffer, 500 μl of 10% HSA, 47°C, 1 hr Incubation

	Yields*	
pH	3-HSA	6-HSA
7.0	63†	95
7.5	49	90
8.0	55	95
9.0	57	—
10.0	60	—
11.0	51	—

* Radiochemical yields based on ^{18}F-labeled **3** or **6** and corrected for decay.
† Incubation period 2 hr.

Labeling of human serum albumin (HSA) with methyl 3-[^{18}F]fluorobenzimidate. The effects of protein concentration, reaction time, and pH on the reaction of [^{18}F]fluorobenzimidate **3** with HSA were carefully studied. The amidination reaction is independent of pH above pH 7.5 (Table 4), but is very dependent on protein concentration (Table 5). The yield of ^{18}F-labeled protein also increases with time (8% at 3 min, 21% at 60 min, and 29.5% at 120 min, 1.2% HSA pH 9.7); longer times than 2 hr were not studied. Yields were determined by HPLC using systems D, E, or F.

HSA labeling with [^{18}F]fluorophenacyl bromide. The alkylation of HSA with p-[^{18}F]fluorophenacyl bromide was also thoroughly examined. Labeling was only slightly dependent on pH (90% at pH 7.0, >95% at pH 8.0 [5% HSA solution, 25°C, 1 hr]), but as shown in Table 5 the labeling efficiency was dependent on protein concentration. A standard incubation period of 1 hr was used for the concentration studies; shorter incuba-

TABLE 5
Dependence of HSA Labeling with ^{18}F-**3** or ^{18}F-**6** on Protein Concentration (100 μl volume, 47°C, pH 8.0, 1 hr)

	Yields (%)*	
% HSA	3-HSA	6-HSA
0.1	—	18
0.2	—	31
0.5	15	75, 82
1.0	30	85, 100
5.0	65	95
10.0	73	—

* Radiochemical yields based on **3** or **6** and corrected for decay.

tion periods are possible with higher protein concentrations (e.g. 5% HSA gives 58% labeling at 5 min, whereas 0.5% HSA yields only 18% labeling after 5 min). The labeling reaction appears not dependent on concentration of 4-[^{18}F]fluorophenacyl bromide, as similar results were obtained using larger volumes of protein solution. Analyses of ^{18}F labeling efficiencies were done by HPLC using system D, E, or F.

Labeling of fibrinogen with methyl 3-[^{18}F]fluorobenzimidate. The reaction of [^{18}F]fluorobenzimidate **3** with purified fibrinogen (conditions: 30μl borate buffer, pH 8.0, 2% fibrinogen, 25°C) gave a low (9.6%) yield of ^{18}F-labeled fibrinogen (HPLC analysis, systems D and F). Increasing the incubation temperature to 36°C raised the yields to 17% (determined by HPLC and as isolated by Sephadex G-25 column chromatography).

Fibrinogen labeling with [^{18}F]fluorophenacyl bromide. The reaction of the α-halo ketone **6** with purified fibrinogen (conditions as above) gave the ^{18}F-labeled fibrinogen in 25–30% yield (HPLC analysis, systems E and F). The [^{18}F]fibrinogen could be isolated by size exclusion chromatography, and retained most (65%) of its ability to form clots when treated with thrombin.

DISCUSSION

Synthesis of methyl 3-[^{18}F]fluoro-5-nitrobenzimidate. Imidates (imido esters) have been previously used for protein modification (*22,23*), including radiolabeling with carbon-14 (*24*) and iodine-125 (*25*). The reaction of imidates with proteins has been shown to be specifically with the ε-amino groups of lysines, forming a stable amidine derivative (*22*). Proteins modified with imidates show no major changes in physical characteristics (electrophoretic mobility, sedimentation coefficient, optical rotation, UV absorbance), indicating minimal disturbance of the net charge or tertiary molecular structure (*26*).

Imido esters were first prepared by acid catalyzed addition of alcohols to nitriles [Pinner reaction, 1892 (*27*)], which yields the imido ester as the hydrohalide (HCl or HBr) salt. We first attempted a Pinner reaction on 4-[^{18}F]fluorobenzonitrile (prepared using 4-nitrobenzonitrile), but found it very difficult to neutralize the excess acid; the free base is needed for efficient protein amidination. Use of the base catalyzed addition reaction (Nef reaction, 1896) (*28*), as in Figure 1, yields the desired free base of the imido ester directly. Reaction of 4-[^{18}F]fluorobenzonitrile with sodium methoxide, however, proved sluggish. As the addition of alkoxides to aromatic nitriles is greatly accelerated by electron withdrawing ring substituents, we chose 3,5-dinitrobenzonitrile **1** as the starting material; the 3-[^{18}F]fluoro-5-nitrobenzonitrile **2** obtained by [^{18}F]fluoride for nitro substitution reacts rapidly and nearly quantitatively with excess sodium methoxide to yield the 3-[^{18}F]fluoro-5-nitrobenzimidate **3** in 17–22% overall yield (isolated, based on total ^{18}F) in an overall synthesis time of less than 1 hr.

The reaction of fluoride ion with 3,5-dinitrobenzonitrile is very interesting. In most nucleophilic aromatic substitution reactions nitro and nitrile groups are considered to be ortho, para-activating (*29*) and in virtually all previous reactions using [^{18}F]fluoride such electron-withdrawing groups were placed ortho or para to the desired leaving group (*19,30–32*). Our success in preparing 3-[^{18}F]fluoro-5-nitrobenzonitrile indicates that two e$^-$-withdrawing groups meta to a good leaving group provide sufficient activation for substitution reactions. The lower yields (42–45%), when compared to reaction of [^{18}F]fluoride ion with p-nitrobenzonitrile (70–80%), indicate that the meta activation is considerably less. A minor peak (R_T = 4 min, system A) may indicate fluoride ion displacement of the nitrile (a poor leaving group) to form 3,5-dinitro[^{18}F]fluorobenzene.

Synthesis of [4-^{18}F]fluorophenacyl bromide. Alkylating agents also have a long history of use as reagents for protein modification (*33*), and alkylation with ^{11}C-methyl iodide is one method for ^{11}C labeling of proteins (*10*). Phenacyl bromide and various substituted phenacyl bromides (p-nitro, p-methoxy, p-azido) (*34–36*) have been previously used for the derivitization of proteins, and carbon-14-labeled p-azidophenacyl bromide has been used in the photoaffinity labeling of an enzyme (*36*). α-Halo ketones are reactive species which can alkylate various heteroatoms (O, N, S) in a protein, and thus can potentially alkylate lysines, tyrosines, histidines, cysteines, and methionines.

The synthesis of 4-[^{18}F]fluorophenacyl bromide was relatively straightforward (Fig. 2). Numerous syntheses of 4-[^{18}F]fluorobenzonitrile have been previously reported by ourselves and others. The reaction of the nitrile with excess organolithium reagent yields the desired [^{18}F]fluoroacetophenone **5**; use of methyl magnesium bromide gave low (<5%) yields, 4-[^{18}F]-Fluoroacetophenone was also prepared by [^{18}F]fluoride ion substitution of 4-nitroacetophenone, but yields of this one-step synthesis were low (5%).

There are numerous methods for the α-bromination of ketones. The bromination of the ketone **5** was done using cupric bromide (*37*) in chloroform-ethyl acetate solution, according to the procedure of King and Ostrum (*15*). After completion of this work Hwang et al. (*38*) reported the bromination of 4-[^{18}F]fluoroacetophenone using bromine-acetic acid in chloroform (reflux). Copper (II) bromide is a convenient reagent, as it is a stable, easy to handle solid; bromination is specific for the methyl groups of acetophenones and the unreacted $CuBr_2$ and by-product (Cu(I)Br) can be removed by simple filtration (in this case through a Florisil SEP-PAK). The rate of bromination of 4-[^{18}F]fluoroacetophenone was studied from 15–60

min, with 30 min chosen as the optimum reaction time (longer times give greater yields, but are offset by nuclide decay). A portion of the 4-[^{18}F]fluoroacetophenone is dibrominated to yield α,α-dibromo-4-[^{18}F]fluoroacetophenone **7**. The percentage of dibromination product increased with longer reaction times and larger amounts of $CuBr_2$. In most cases the reaction of ketone **5** with 50 mg $CuBr_2$ for 30 min (reflux) gave a mixture of desired phenacyl bromide **6** (85–90%), unreacted ketone (5–7%) and dibromination product **7** (5–7%). The unreacted ketone **5** is effectively removed in the evaporation steps, as it exhibits much greater volatility than the brominated ketones. Yields are excellent (85–90% corrected), and the product was deemed suitable for protein labeling without further purification.

Overall isolated yields of [^{18}F]fluorophenacyl bromide are from 28–40% (decay corrected) in a synthesis time of 75 min.

Specific activity. Although the syntheses of the imidate **3** (Fig. 1) and α-bromoketone **6** (Fig. 2) were done using high specific activity, no-carrier-added [^{18}F]fluoride ion, HPLC analysis of the final products indicated UV-absorbing mass eluting close to, or coincident with, the radioactive peaks. They are most likely due to the formation of methyl 3,5-dinitrobenzimidate and 4-nitrophenacyl bromide, respectively: co-injection studies of the corresponding fluoro- and nitro-compounds indicated they could not be separated under the analytical HPLC conditions used. These nitro compounds can be considered as "pseudo" carrier compounds, as they could be expected to also participate in protein modification reactions. Although specific activity is not usually a consideration in protein labeling, the use of a ^{18}F labeling reagent containing a large amount of such "pseudo" carriers could result in modification of multiple amino acid residues per protein. Fortunately, in both the synthesis of the imidate **3** and the α-bromoketone **6**, not all of the starting materials (1 mg of a nitrobenzonitrile in each case) is carried through the synthesis. For the benzimidate **3** we estimate the final product contains 200–300 μg of the 3,5-dinitrobenzimidate. In the synthesis of 4-fluorophenacyl bromide (Fig. 2), most of the nitrobenzonitrile is destroyed by the large excess of methyllithium used in the second step, resulting in a final product containing less than 100 μg of 4-nitrophenacyl bromide. These amounts of cold material apparently do not, in the proteins studied here, alter the protein structures to a significant degree. For truly no-carrier-added ^{18}F-labeled reagents some method of separation of nitro and fluoro compounds would be needed. It should be noted, however, that the dinitrobenzimidate and 4-nitrophenacyl bromide are both likely to be much more reactive than their fluorine-containing counterpoints, and the relative rates of protein modification versus hydrolysis have not been determined.

Protein labeling. Both 4-[^{18}F]fluorophenacyl bromide and methyl 3-[^{18}F]fluoro-5-nitrobenzimidate have been used to successfully attach ^{18}F to proteins. The influence of reaction conditions was studied using HSA as the model protein. Neither ^{18}F-labeled reagent exhibited any dependence on pH (Table 4), but both showed a dependence on protein concentration (Table 5). All yields were determined using a standard one-hour reaction time, but shorter reaction times are possible at higher protein concentrations, particularly with the α-bromo-ketone. In all cases, there was no dependence of yield or reaction rate on the concentration of ^{18}F reagent (**3** or **6**), as nearly identical results were obtained using larger reaction volumes.

The stability of the ^{18}F-fluorobenzimidate **3** in aqueous solution at pH 8 is somewhat surprising in view of previous observations of rapid imido ester hydrolysis (*39*). Previous workers have recommended use of higher pH values (pH 10) to effect faster amidination in the presence of such hydrolysis reactions; higher pH solutions, however, are not desirable for protein labeling. That 100% protein labeling is not achieved, even in very concentrated HSA solutions, may be indicative of competition between amidination and hydrolysis at long reaction times.

HSA labeled with **3** or **6** could be conveniently isolated by size exclusion chromatography. In the in vivo studies of isolated ^{18}F-labeled HSA, both the percent injected doses per gram (blood) and blood clearance rates were very similar to ^{125}I-labeled HSA (Fig. 3). These preliminary results suggest that for HSA the radiolabeled protein maintains its biologic integrity, and protein metabolism does not release ^{18}F-metabolites which accumulate in the blood.

As HSA is not easily denatured it is not a good model for all proteins of possible interest; we therefore also studied the covalent attachment of [^{18}F]fluorophenacyl bromide **6** and [^{18}F]fluoroimidate **3** to human fibrinogen. Fibrinogen is a protein which can be readily denatured by elevated temperatures, and by procedures used for isolation, radiolabeling, and storage (*40*). The reaction of fibrinogen with [^{18}F]fluoroimidate **3** gave low (17%) yields of [^{18}F]fibrinogen, probably due to the low concentration (2%) of protein utilized in the reaction. Reaction of fibrinogen with 4-fluorophenacyl bromide gave better yields (25–30%) of the radiolabeled fibrinogen. Both ^{18}F-labeled fibrinogens could be isolated by size exclusion chromatography, and retained most (60–65%) of the ability to clot when treated with thrombin. Previous workers have suggested, however, that clottability is a convenient laboratory test but may not be a definitive measure of the viability of radiolabeled fibrinogens (*40*).

Finally, preliminary results in the labeling of an immunoglobulin (human IgA) indicate that either the fluoroimidate **3** or α-bromoketone **6** may prove useful

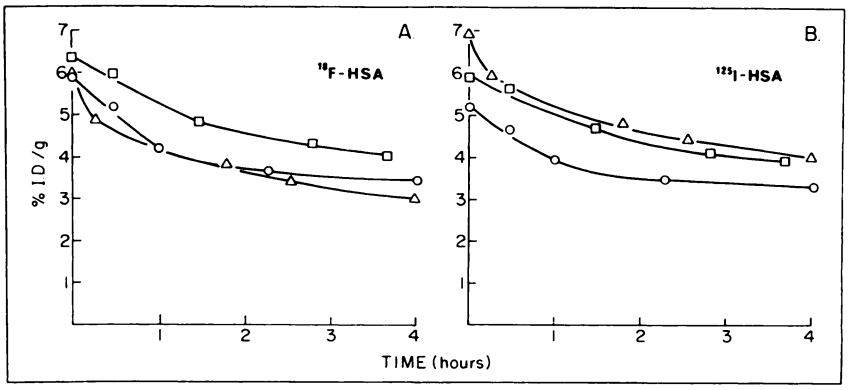

FIGURE 3
Blood concentrations of [^{18}F]-6-HSA and [^{125}I]HSA in rats at times up to 4 hr postadministration. Shown are data for three rats (○, □, △). Identical results were obtained with [^{18}F]-3-HSA.

for ^{18}F labeling of antibodies. The yield of this reaction is also very concentration dependent (Table 6). As antibodies are often precious commodities, development of micro techniques to minimize reaction volumes will be necessary. There has been extensive work in the radiolabeling of antibodies with radioiodine and metal radioisotopes, but we believe this is the first approach to ^{18}F labeling.

The two reagents prepared here, 4-fluorophenacyl bromide and methyl 3-fluoro-5-nitro-benzimidate, offer differing reactivities toward proteins, with the α-bromo ketone **6** in general more reactive and producing higher yields. The alkylating agent, however, is a much more indiscriminate label than the less reactive but selective (for lysine amino groups) methyl imidate. We have not attempted to identify those amino acid residues modified by either reagent. Limited studies using mercaptoethanol, however, have demonstrated that 4-[^{18}F]fluorophenacyl bromide is not reacting with HSA through reversible aklylation of methionines (to form alkylsulfonium salts). The choice of reagent, fluoroimidate **3** or fluorophenacyl bromide **6**, would most likely be made, not on the basis of higher radiochemical yield, but rather on which reagent gave the least pertubation of the biological properties of the protein under study.

Using either 4-[^{18}F]fluorophenacyl bromide or methyl 3-[^{18}F]fluoro-5-nitrobenzimidate, we have prepared modest amounts (100–1000 μCi) of ^{18}F-labeled proteins for small animal studies. Production of larger amounts (5–10 mCi) will necessitate use of higher initial levels of radioactivity ([^{18}F]fluoride ion) and, most likely, remote or automated apparatus. These constraints should be easily overcome, as we have previously reported a simple method to produce large amounts of [^{18}F]fluoride ion (*16*), and have described the application of robotics to the automated preparation of several ^{18}F- and ^{11}C-labeled radiopharmaceuticals (*41*).

Finally, the two reagents reported here (^{18}F-fluoroimidate **3** and ^{18}F-fluorophenacyl bromide **6**) may have applications in biochemical studies of proteins using ^{19}F NMR spectroscopy, and would complement reagents [ethyl thioltrifluoroacetate (*42*), p-fluorobenzenesulfonyl chloride (*43*), and 3,3,3,-trifluorobromoacetone (*44*)] which are currently in use.

Methyl 3-fluoro-5-nitrobenzimidate and 4-fluorophenacyl bromide should prove useful reagents for ^{18}F or ^{19}F labeling of a wide variety of proteins. In more recent studies we have successfully ^{18}F labeled human transferrin, hemoglobin, red blood cells, platelets, and HSA microspheres, and also F(ab')$_2$ fragments of a mouse antibody. Efforts in ^{18}F labeling of these and other proteins is continuing.

TABLE 6
Labeling of Human Immunoglobulin A (IgA) with ^{18}F Labeling Agents **3** and **6** (0.033M Borate Buffer, pH 8.0, 47°C, 1 hr)

	Yields (%)*	
% IgA	3-IgA	6-IgA
0.66	9.1	19
2.2	15.6	46

* Radiochemical yields based on **3** or **6** and corrected for decay.

NOTES

* Aldrich Chemical Company, Milwaukee, WI.
† Sigma Chemical Corp., St. Louis, MO.
‡ Waters Associates, Milford, MA.
§ Mallenckrodt, Inc., St. Louis, MO.

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