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A GENERAL METHOD FOR LABELING PROTEINS WITH ^{11}C

Maria G. Straatmann and Michael J. Welch

Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, Missouri

A method for labeling proteins with ^{11}C has been developed. The method consists of adding ^{11}C-formaldehyde (derived from $^{11}CO_2$ by catalytic oxidation of ^{11}C-methanol) to a buffered protein solution, followed by reduction of the resulting formaldehyde-protein adduct with aliquots of sodium borohydride. Separation of the desired product is achieved simply and efficiently using high-speed liquid chromatography or, in the case of fibrinogen, by ammonium sulfate precipitation. This mild technique leads to high yields of labeled protein and is adaptable to the labeling of proteins and peptides or other molecules which contain a free amino group and which are stable at pH 8.

Carbon-11 with its 20.4-min half-life is a convenient, positron-emitting isotope, ideal for use in in vivo tracer studies. Much work has been done recently on incorporating the most common ^{11}C-containing precursors into more complicated compounds (*1–7*). The problem becomes one of developing rapid, efficient syntheses requiring a minimum of complexity to achieve labeling within the time limits of the short-lived ^{11}C. We have attempted to develop a general method for labeling proteins with ^{11}C due to our interest in compounds labeled with short-lived, positron-emitting isotopes and particularly in a blood protein label for use in conjunction with ^{15}O-carboxyhemoglobin to measure regional brain hematocrit (*8*).

A method for attaching methyl groups to protein amino groups by reductive alkylation has been described by Means and coworkers (*9,10*). In their method an excess of formaldehyde was added to the protein in a pH 9 buffer followed by sequential additions of sodium borohydride to reduce the formaldehyde adjunct to a methyl group. Using this technique and excess ^{14}C-formaldehyde, they developed a labeling procedure that exhibited 20% of the maximum labeling efficiency and a 4–5% yield based on the ^{14}C-formaldehyde used. This procedure allows the gentle introduction of an isotope with minimum alteration of the protein structure. The current work involves an adaptation of this method using ^{11}C-formaldehyde and a variety of substrates. Maziere, et al reported an ^{11}C-chlorpromazine preparation involving methylation of nor-1-chlorpromazine using ^{11}C-formaldehyde in the presence of formic acid (*7*). Their method gave a 14% labeling efficiency based on incorporation of the available label.

METHOD

Preparation of ^{11}C-formaldehyde. Preparation of $H^{11}CHO$ in our laboratory is based on the method of Wolf, et al (*4*). The $^{11}CO_2$ was prepared using the 7-MeV deuteron beam of the Washington University Medical School cyclotron by bombarding boric oxide in an atmosphere of helium containing 3% oxygen. The ^{11}C formed by the $^{10}B(d,n)^{11}C$ nuclear reaction reacts with oxygen in the boric oxide and is released as carbon monoxide, which undergoes radiolytic oxidation to carbon dioxide (*11*). At the end of an 18-min irradiation, the gas was trapped in liquid nitrogen. The $^{11}CO_2$ was then flushed with helium into an evacuated chamber for the subsequent conversion to $^{11}CH_3OH$. Lithium aluminum hydride* (0.2 cc of ~ 1 *M* in ether, Alfa Inorganics) and 2 cc anhydrous ether were added to the chamber under nitrogen and the vessel was shaken for 2 min. Using 1 cc 1.0 *M* HCl, the excess hydride was destroyed and the sample hydrolyzed. The ether was then evaporated under reduced pressure.

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For reprints contact: Michael J. Welch, Mallinckrodt Institute of Radiology, Washington University School of Medicine, 510 S. Kingshighway, St. Louis, Mo. 63110.

* As pointed out in the discussion, small amounts of CO_2 from the air give rise to competing unlabeled formaldehyde molecules. To minimize the amount of carrier atmospheric carbon dioxide absorbed in the solution, immediately on receipt of the hydride from the manufacturer, it was divided into several small aliquots, each stored under nitrogen in brown glass bottles. Samples were withdrawn from each for only a few experiments.

For catalytic oxidation to $H^{11}CHO$, the sample ($\sim$ 1 cc) was introduced to the catalytic furnace through a preheated injector. A constant flow of oxygen (0.5 cc/min) flushed the furnace which was held at 375°C and contained an iron-molybdenum catalyst. The catalyst was prepared by the method of Adkins and Peterson (*12*) using $\frac{3}{16}$-in. diam stainless steel balls as a support. After passing through the furnace, the $H^{11}CHO$ was trapped directly in an ice-cooled flask containing pH 9.0 borate buffer (0.2 *M*). Careful attention must be paid to the pH of the solution at this point. As more and more injections are made on the furnace, HCl gas tends to adsorb on the furnace surfaces to be slowly released, causing alteration of the pH in the receiving flask. This problem can be overcome by using a nonvolatile acid such as sulfuric acid for the hydrolysis. This mixture was then distilled into 0.2 cc pH 8 borate buffer (0.2 *M*).

The distilled solution was monitored for percent conversion from methanol to formaldehyde by radio-gas partition chromatography. The amounts of $^{11}CH_3OH$ and $H^{11}CHO$ were determined using an 8-ft stainless steel column packed with Poropak Q at 90° in a Perkin-Elmer 820 chromatograph. An internal flow proportional counter interfaced to a classic LINC computer that was programmed to correct for radioactive decay, background, and flow rate variations (*13*) was used in conjunction with the chromatograph.

The average oxidation yield as determined by the gas chromatography is $\sim$ 75%. Yields vary according to age of catalyst and rate of injection. The catalyst is good for about 25 injections before the furnace must be repacked.

Protein labeling. Since we were interested in this project as a general protein-labeling procedure, two model compounds were studied: albumin to optimize yields and investigate the reaction and fibrinogen to evaluate this labeling method on a protein for which a simple test of its biologic activity is available. The basic reaction mixture contained 200 μl ^{11}C-formaldehyde solution and 100 μl of protein solution containing between 0.5 and 10 mg of protein in borate buffer. This mixture was allowed to equilibrate for 2 min and 10 μl of 0.1 *M* sodium borohydride was added. The borohydride solution was prepared by diluting a solution of sodium borohydride in sodium hydroxide (Ventron Corp., Beverly, Mass.) with the borate buffer. After addition of the borohydride, the protein was either rapidly separated from the solution or the pH was adjusted to between 5.5 and 6.5 with dilute hydrochloric acid to decompose the excess sodium borohydride. All reactants were varied over a range of one fourth to ten times the original concentrations to study the amounts of the reactant delivering the best yield, and the pH was varied from 7 to 10 to study its effect.

Analysis and purification of the albumin was carried out by high-pressure liquid chromatography using a 2 ft $\times$ $\frac{3}{8}$-in. column with a Waters Associates Model 202 liquid chromatograph using deionized distilled water as the eluting solvent at a flow rate of 2.5 ml/min. Column packings used were Hydrogel IV (Waters Associates, Milford, Mass.), Corning C.P.G. glass beads (CPG-10-75), and Glycophase-G/CPG-100-coated glass beads. The eluate was passed over the face of a scintillation crystal and the signal from the single-channel analyzer was interfaced to the LINC computer for data collection and correction. Trichloroacetic acid (TCAA) precipitations were carried out on the solution to confirm the yield values.

The fibrinogen was purified by a single precipitation using 30% ammonium sulfate. After dissolution of the separated precipitate, TCAA precipitation and clottability measurements were carried out, the latter using the method of Regoeczi (*14*).

RESULTS

Variation in pH showed that the yield of labeled product dropped by an order of magnitude as the pH was decreased from 8 to 7. On increasing the pH from 8 to 10 a slight decrease in yield was observed. Production runs were therefore carried out at pH 8. Increasing the amount of $NaBH_4$ from the 10 μl of 0.1 *M* (1 μ*M*) sodium borohydride did not increase the yield, so 1 μ*M* was used per 0.2 ml of formaldehyde. Variation in ^{11}C-albumin yield with amount of albumin is shown in Fig. 1. Good fast separation of the protein and residual methanol and formaldehyde were only obtained using the Hydrogel column; the other columns used did not completely separate the material nor give such a sharp protein peak. A typical chromatogram of the labeled albumin on Hydrogel is shown in Fig. 2. In this and subsequent preparations, yields of 35–43% based on total activity added to the protein were obtained. The mean yield was 38.6%, which corresponds to a yield of 52% based on available formaldehyde. The production of the formaldehyde took approximately 10 min and the distillation, synthesis, and purification approximately 20 min. Approximately 70% of the activity was recovered in both the formaldehyde preparation and distillation procedure. After chromatography, from 3 to 5 mCi of final product in a volume of $\simeq$ 7 cc of water were obtained from an

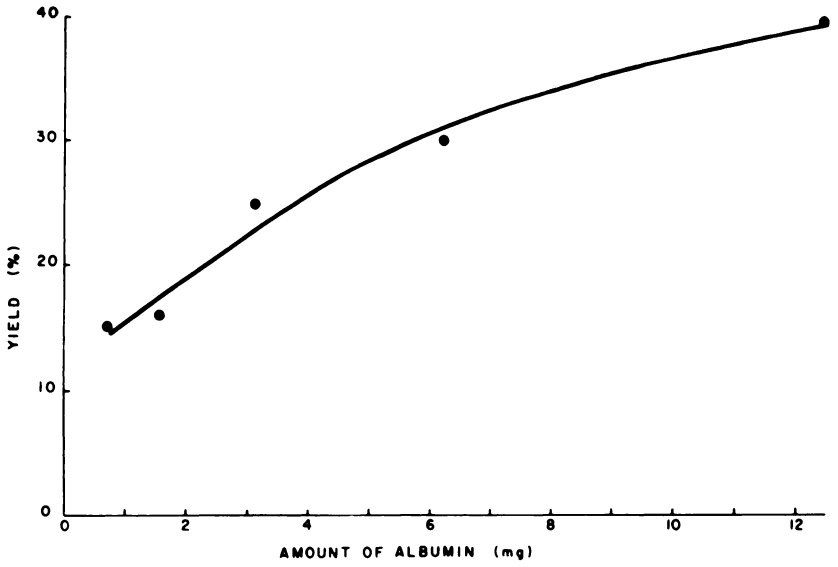

FIG. 1. Variation in yield of ^{11}C-methyl albumin with concentration of albumin used. Yields are based on total activity available and all other parameters other than albumin concentration were identical.

initial activity of 30–40 mCi trapped from the cyclotron target.

The fibrinogen labeling was carried out on 0.2 ml of solution containing 12 mg protein/ml. Ammonium sulfate and TCAA precipitation gave yields ranging from 26 to 41% (mean, 33%) based on available ^{11}C-formaldehyde. Clottability studies exhibited a mean clottability of 85 ± 8%.

DISCUSSION

High yields of ^{11}C-labeled proteins have been obtained by the reduction of the formaldehyde-protein complex, with consistent yields obtained by three different techniques. Presumably the reaction sequence in the labeling procedure is:

$$R\text{–}NH_2 + H_2{}^{11}CO \rightleftarrows R\text{–}\underset{\displaystyle H}{\overset{\displaystyle OH}{N}}\text{–}{}^{11}CH_2 \xrightarrow{NaBH_4} R\text{–}\underset{\displaystyle H}{N}\text{–}{}^{11}CH_3.$$

Means, et al (*9,10*) have shown that this type of methylation is very mild and affects the biologic activity of a protein less than conventional labeling methods.

We have shown that fibrinogen labeled with this method is still highly clottable. Fibrinogen is probably a good model compound for such studies since terminal groups presumably will label preferentially and terminal groups are known to be cleaved in the

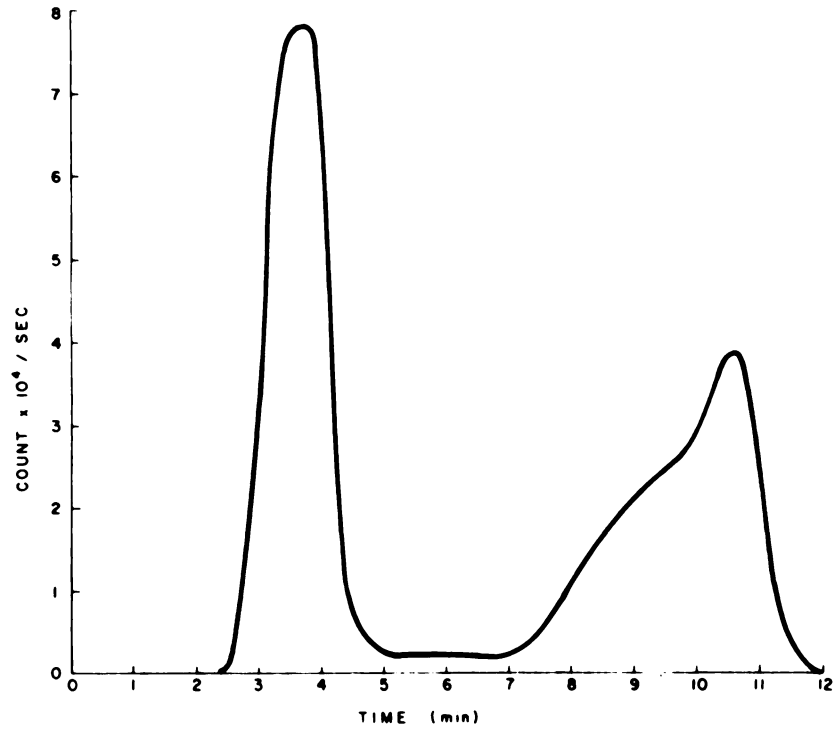

FIG. 2. Chromatogram of ^{11}C-methyl albumin reaction mixture. The $^{11}CH_3$-albumin elutes first and second peak is ^{11}C-methanol/formaldehyde. Hydrogel IV column was used at pressure of ≃ 250 lb.

fibrinogen-to-fibrin transformation. Despite this, the isotopic clottability is similar to that of conventional iodinated preparations (*15*).

The yields obtained are sufficient for most tracer applications and are considerably higher than those of Rice and Means (*10*), who used excess ^{14}C-formaldehyde, and Maziere, et al (*7*), who used ^{11}C-formaldehyde. A method of preparation in which the protein is purified by high-pressure liquid chromatography in only 5 min has been developed. The highest yields occur with a large amount of protein, but decreasing the amount of protein by an order of magnitude only decreased the yield to $\sim 40\%$ of the original yield. This is, of course, important if one is interested in labeling expensive or slightly soluble proteins. The results obtained with albumin shown in Fig. 1 predict a yield of $\simeq 21\%$ with 2–4 mg of protein based on amount of total activity, which is equivalent to a yield of 28% based on available ^{11}C-formaldehyde. In experiments involving fibrinogen, a mean yield of 33% was obtained showing that the proteins studied gave similar yields at similar concentrations.

The yield is presumably limited by the amount of the intermediate A in the following reaction present when the reducing agent is added:

$$R\text{–}NH_2 + H_2{}^{11}CO \rightleftarrows R\text{–}\underset{\underset{A}{|}}{\overset{\overset{H}{|}}{N}}\text{–}{}^{11}CH_2\overset{OH}{|}$$

A greater amount of protein in solution will force the equilibrium to the right and tend to give a greater yield at greater protein concentrations.

Although increasing the amount of formaldehyde will have the same effect, there are only a limited number of labeling sites available on each protein and care needs to be taken to keep the concentration of carrier formaldehyde low. Rice and Means (*9*) estimate that $\simeq 1\ \mu M$ of formaldehyde will convert all the protein-free amino groups into the dimethyl amino derivative. This amount will, of course, vary from protein to protein. One μM of formaldehyde is derived from carbon dioxide which is contained in approximately 2.5 cc of air. This emphasizes the need to use small amounts of $LiAlH_4$ that have had as little contact with air as possible, thus containing a minimum of absorbed CO_2 to avoid competition with labeled formaldehyde for the available amine sites.

In conclusion, a mild method for ^{11}C-labeling of proteins has been developed and should be applicable for the ^{11}C methylation of any protein, peptide, or compound that contains a free amino group and that is stable under the mild conditions used. Possible applications involve the labeling of bleomycin for tumor detection and urokinase for clot detection since both of these compounds have very short biologic half-lives (*16,17*).

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