SYNTHESIS, ANALYSIS, AND STABILITY STUDIES OF ^{14}C-METHYL(ACETOXYMETHYL)NITROSAMINE

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SUMMARY

The one-step synthesis of ^{14}C-methyl(acetoxymethyl)nitrosamine (^{14}C-DMN-OAc) of high specific activity is described. The product was shown to decompose by hydrolysis, as reported previously for the unlabelled material, and by self-radiolysis, a process which occurred even in methylene chloride solution at high specific activities. Both modes of decomposition led to disappearance of label from the solution, at least partly by sorption to the glass storage vessel. Thin-layer chromatography proved less suitable for resolving the decomposition products than radio-gas chromatography. Labelled carbon atoms were found to the extent of only 1% in the formaldehyde produced on esterase hydrolysis of ^{14}C-DMN-OAc, showing that scrambling during the synthesis was not extensive. Storage of aqueous solutions for biological work was best accomplished in frozen aqueous solutions of pH 5.5.

Key Words: Nitrosamine, autoradiolysis, labelled carcinogen

INTRODUCTION

The synthesis (1,2) of methyl(acetoxymethyl)nitrosamine (DMN-OAc), a stable ester derivative of the presumed active metabolite of the potent carcinogen, dimethylnitrosamine, has led to a variety of important studies on the biological consequences of exposure to N-nitroso compounds (3).

The purpose of this paper is to provide full details of our preparation of ^{14}C-methyl(acetoxymethyl)nitrosamine (^{14}C-DMN-OAc), which involves a synthetic approach somewhat different from that previously reported by Braun and Wiessler (4). We also wish to summarize our observations and recommendations regarding the purification, analysis, decomposition, and storage of this important biological research tool.

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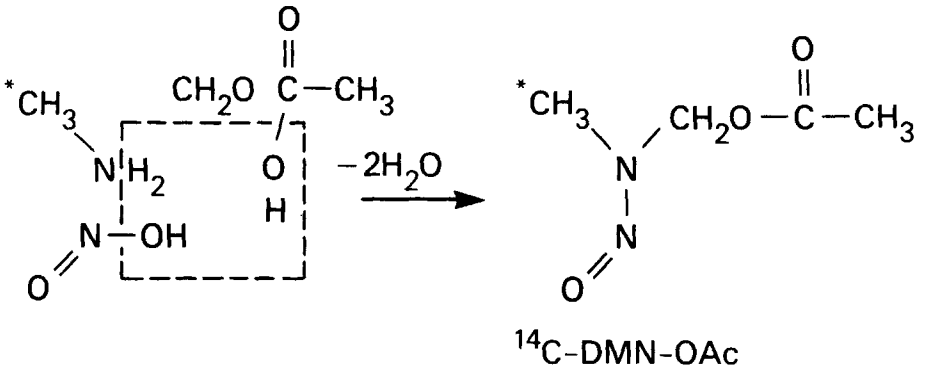

Figure 1. Synthesis of ^{14}C-methyl(acetoxymethyl)nitrosamine (^{14}C-DMN-OAc).

RESULTS AND DISCUSSION

Synthesis. - Our preparative method, which was adapted from a previously reported synthesis of the cold material (2), involves the condensation of carbon labelled methylamine hydrochloride with formaldehyde, acetic acid, and sodium nitrite in the presence of minimal water, as outlined in Figure 1.

The synthetic procedure was carried out entirely in one reaction vessel, leading to what we feel is the considerable advantage of reducing the potential hazard of the preparation by minimizing the number of manipulations of the labelled carcinogen. On the other hand, the yields in our procedure were somewhat lower than the 24% conversion reported by Braun and Wiessler (4). We normally obtained yields of 13-15% after distillation of the ^{14}C-DMN-OAc; however, one preparation in which the product was isolated by extraction into methylene chloride with no further purification resulted in a 27% conversion, suggesting that losses during our evaporative distillation step were substantial.

Stability Studies. - Two different decomposition pathways were observed when freshly prepared ^{14}C-DMN-OAc was stored.

One was autoradiolysis of highly radioactive material. One preparation

was dissolved in methylene chloride at a concentration of about 1 mCi/mL and stored in a sealed glass ampule for 20 months. Subsequent analysis revealed that the sample suffered 90% decomposition during that time. About 10% of the original counts were found to be contained in a yellowish methylene chloride-insoluble gum which deposited in the ampule. Of the roughly 20% of the original counts which remained in solution, most were associated with impurities (see Analysis section below). It is not clear what happened to the remaining 70% of the original counts, but a substantial portion (perhaps all) was sorbed to the glass, as determined by scintillation counting of the crushed ampule. A small portion of the glass drove the counter offscale. We were unable to properly quantify the proportion of label sorbed to glass, due to difficulties involved in taking sufficiently small aliquots of such an inhomogeneous sample.

The other decomposition pathway we observed was hydrolysis, which at least at low specific activities closely paralleled our earlier (2) experience with unlabelled DMN-OAc. The half-life for decomposition of a 20 mM solution containing 0.5 uCi/mL in pH 5.5 buffer at 21° was 19 days, compared to 20 days in pH 5.1 buffer for the cold compound. The disappearance rate for the cold unlabelled molecules, as measured by following the absorbance at 352 nm, was the same as that of the ^{14}C-containing molecules in this dilute solution, as determined by integrating the radio-gas chromatography peak corresponding to ^{14}C-DMN-OAc.

Fortunately, the rate of solvolytic decomposition in aqueous buffer (the medium of choice for the biological experiments the material would be subjected to) was largely controllable by varying the temperature of storage. The first order hydrolysis rate constant fell by an order of magnitude when the temperature of a low-activity preparation (0.5 uCi/mL) was dropped from 21°C to 1°C, with even slower decomposition being observed in frozen solutions (see Table 1).

Clearly, cold storage is to be recommended for these preparations, preferably in a deep freeze.

Table 1. First-order rate constants for decomposition of ^{14}C-DMN-OAc in pH 5.5 buffer.

	Storage temperature (°C)				
	+21°	+1°	-18°	-56°	Specific activity
k for loss of absorbance at 352 nm (day^{-1})	0.0375	0.0039	0.0024	0.0014	0.5 uCi/mL
k for loss of ^{14}C from solution (day^{-1})	-	-	0.004	-	740 uCi/mL

Interestingly, the hydrolysis was also accompanied by substantial loss of label from solution. One highly radioactive frozen sample originally containing 0.74 mCi/mL lost 29% of its counts from solution during storage in pH 5.5 buffer at -18°C for 84 days. Thus, the loss of ^{14}C from solution at this high specific activity seemed to be as fast as (or possibly significantly faster than) ester hydrolysis was in low-activity solution (see Table 1). This suggests not only that a second, nonhydrolytic pathway (presumably autoradiolysis similar to that described above) is contributing to the overall decomposition of ^{14}C-DMN-OAc in aqueous solution, but also that both autoradiolysis and hydrolysis proceed with loss of at least some radioactivity from the solution. We had previously noted that the cold material's methyl group could not be quantitatively recovered as methanol during hydrolysis in alkaline solution (2). It seems reasonable to conclude that the methyl groups of ^{14}C-DMN-OAc are lost from aqueous solution during hydrolysis as well as during autoradiolysis by sorption to the glass, as seemed to have happened during autoradiolysis in methylene chloride solution (see above).

However, other pathways (e.g., formation and evaporation of volatile dimethyl ether) cannot be ruled out at this time.

Analysis. - The freshly distilled product had a chemical and radiochemical purity of approximately 95%, as determined by gas and thin-layer chromatography, respectively. Most of the other information we obtained on the purification and analysis of ^{14}C-DMN-OAc was consistent with expectation, but two of our findings are worthy of separate mention.

One is that the product mixtures resulting from the extensive decomposition reactions described in the previous section seemed to be more easily resolved by gas chromatography using a gas proportional radioactivity counter than by thin-layer chromatography. A typical radio-gas chromatogram is shown in Figure 2a; nine different major radioactive peaks were found. By contrast, only three components could be resolved in the thin-layer chromatogram whose manual radioscan is shown in Figure 2b. It should be pointed out that, although the same autoradiolyzed sample was used for both chromatography experiments of Figure 2, the gas chromatogram was run on material which had been transferred to aqueous pH 5.5 buffer and partially hydrolyzed before injection, while the thin-layer work was done directly on the methylene chloride solution before transfer to aqueous buffer; thus, the two chromatograms are not entirely comparable. Nevertheless, we conclude that thin-layer chromatography on silica gel had insufficient resolving power to separate many contaminants from each other and from the ^{14}C-DMN-OAc. In support of this conclusion, thin-layer radioscans run after the sample had been transferred to aqueous buffer and partially hydrolyzed showed sometimes one, sometimes two labelled components in the region of the DMN-OAc fluorescence-quenching spot, depending upon how the chromatogram was cut for scintillation counting. The maximum number of resolved components still did not exceed three, however, since the radioactive fraction at the solvent front disappeared when the sample was transferred to aqueous buffer from methylene chloride solution.

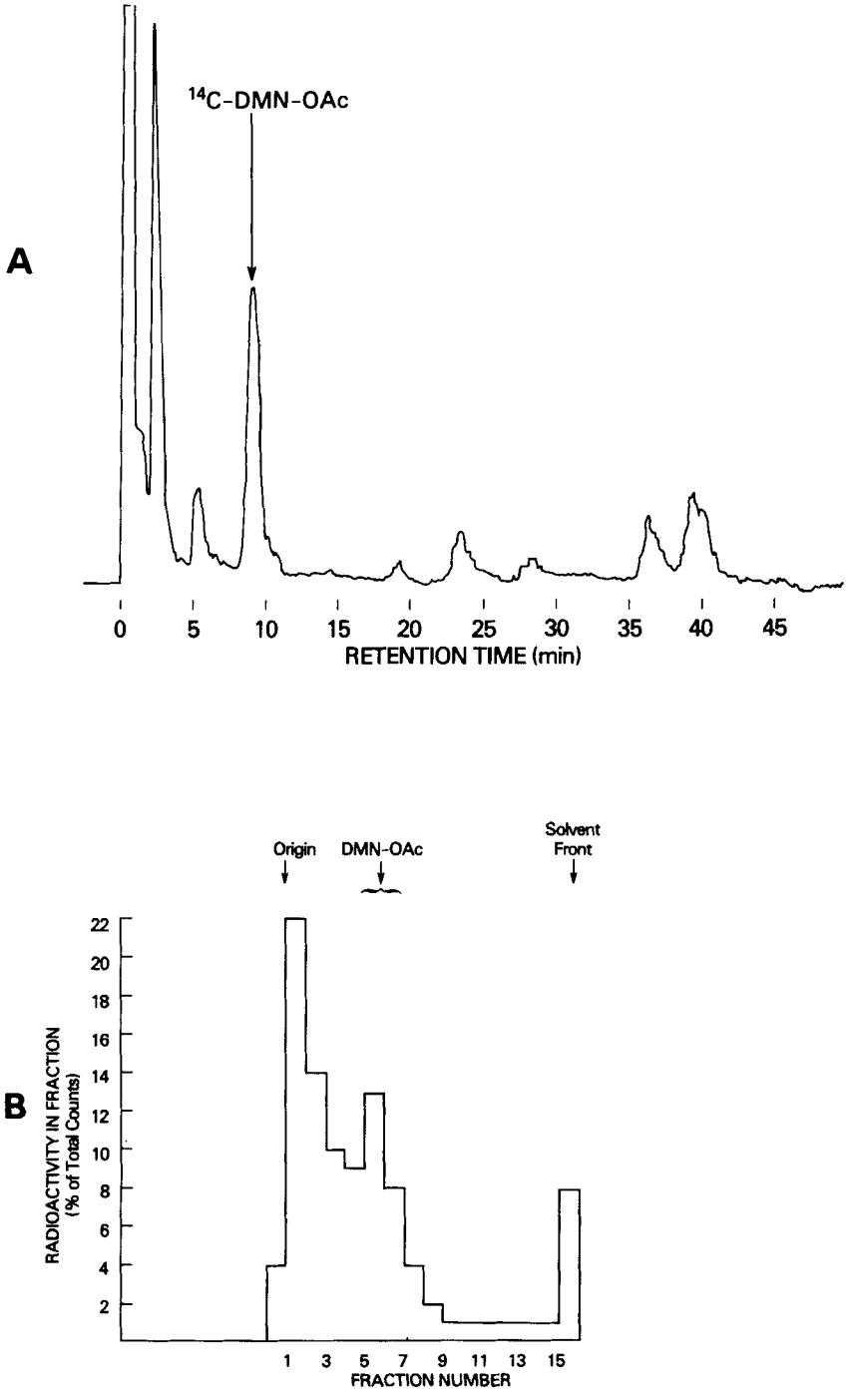

Figure 2. Analysis of autoradiolyzed ^{14}C-DMN-OAc:

A) by radio-gas chromatography;
B) by manual radioscan of a thin-layer chromatogram.

The second noteworthy point is that the carbon label seems to have little or no tendency to scramble away from the N-methyl group. If the methylene and methyl carbon atoms became equivalent at some stage during synthesis of ^{14}C-DMN-OAc, the resultant label scrambling would have to be taken into account in interpreting biological experiments in which these preparations were used. We tested for this possibility by deliberately hydrolyzing an aliquot of ^{14}C-DMN-OAc using hog liver esterase catalysis and isolating the formaldehyde thus produced as the dimedone adduct. While the formaldehyde fraction was found to contain analytically significant radioactivity, the quantity corresponded to only about 1% of the total ^{14}C available. We conclude that this level of scrambling is probably too low to complicate interpretation of most biological results, but we recommend that the findings be borne in mind when evaluating future experiments.

EXPERIMENTAL

Synthesis. - The synthesis of ^{14}C-DMN-OAc was carried out four times, at activity levels of 0.26, 55, 49, and 62 mCi/mmole. The most recent preparation, described in the next paragraph, is illustrative.

^{14}C-Methylamine hydrochloride was purchased from Amersham/Searle at a specific activity of 61.9 mCi/mmole. Fifty milliCuries (56.2 mg) was mixed in a 10 mL flask with 30 mg of paraformaldehyde, 4.3 mL of glacial acetic acid, and 50 uL of water. The flask was equipped with a rubber septum containing a large bore hypodermic needle which served as a vent. The solution was kept at 60° for 30 minutes until all solids were dissolved, then cooled in an ice bath at 10° for 1 hour during the addition of 0.8 mL of 1.0 M sodium nitrite. The solution was warmed to room temperature, stirred overnight, poured into a 100 mL flask, and treated with 70 mL of cold, saturated sodium bicarbonate solution, bringing the pH to 7. The aqueous solution was extracted with three 15

mL portions of methyene chloride. The combined organic extracts were washed with two 3 mL portions of water, dried with magnesium sulfate, and evaporated at 20 mm pressure, first at 0° until most of the solvent was gone, and then at 20° for 10 minutes. The remaining liquid was evaporatively distilled at 20 mm and 80°. The yield was estimated to be 16.5 mg (15%) by dissolving the distillate in 50 mL of hexane and measuring the ultraviolet absorbance at 227 nm, assuming a molar absorptivity of 7200. Scintillation counting yielded a specific activity value of 62 mCi/mmole.

The ^{14}C-DMN-OAc was placed in phosphate buffer for biological work by evaporating the hexane solution prepared in the previous paragraph at 0°, then dissolving the residue in 7.6 mL of pH 5.5 phosphate buffer which was 0.15 M in sodium ion. Scintillation counting revealed that only 12 mg of nitrosamine remained in the aqueous phase. An additional 4.4 mg was found by ultraviolet spectrophotometry to have co-distilled with the hexane, and was located in the dry ice trap.

Analysis and purification. - Chemical purity of the freshly prepared, distilled ^{14}C-DMN-OAc was determined by gas chromatographic analysis of its hexane solution on a 9.3 m glass capillary column of SP-2100 programmed from 60-200° at 20°/min. Nitrogen flow rate was 22 mL/min. With a flame ionization detector, 6 impurities each integrating for 0.36-1.51% of the total peak area of the chromatogram could be detected at retention times ranging from 1.79 to 4.00 min; ^{14}C-DMN-OAc had a retention time of 2.53 min, and integrated for 94.5% of the total peak area.

The radiochemical purity of freshly purified ^{14}C-DMN-OAc was measured by thin-layer chromatography on precoated glass plates of Silica Gel 60-F254 (E. Merck) or glass Silica GF-254 plates (Analtech) with 1:1 diethyl ether:hexane

as developer. Using ultraviolet quenching and photographic film for detection, the major spot (95% of total activity) was found at an R_f ranging in the various experiments between 0.25 and 0.66, with a minor, ultraviolet-absorbing, radioactive spot containing 5% of the total activity at an R_f value 60% of that of the DMN-OAc. A minor spot also occasionally appeared at the origin.

For radio-gas chromatography of the partially decomposed samples, a Packard-Becker 409 Gas Chromatograph equipped with a Packard Model 894 Gas Proportional Counter and a 6' x 1/8" outer diameter stainless steel column of 15% diethylene glycol succinate on 80/100 Chromosorb WAW-DMCS at a column temperature of 140° and a helium flow rate of 40 mL/min was used for direct injection of aqueous buffer solutions in 10 uL aliquots. By comparing the response given by the radio-gas chromatography detector with that given by a thermal conductivity detector located between the column and the radio-gas chromatography detector, ^{14}C-DMN-OAc was shown to have a retention time of 7.5 minutes under these conditions. The ^{14}C-DMN-OAc was quantitated by tracing its peaks onto paper of uniform thickness and weighing their cutouts. By injecting samples subjected to varying degrees of hydrolysis, then comparing the peak areas determined in this way with the concentrations of cold material measured by ultraviolet absorbance at 352 nm, the concentration of ^{14}C-DMN-OAc was shown to be directly proportional to that of the unlabelled carcinogen in these samples. A typical radio-gas chromatogram of an autoradiolyzed, partially hydrolyzed sample is shown in Figure 2a.

For thin-layer chromatographic separations of the methylene chloride soluble portion of a similar sample, Silica Gel 1B-F plastic backed TLC Plates (Baker-flex) were used with methylene chloride as developer. The resulting chromatograms were cut into 5 mm fractions, each of which was dropped into a scintillation vial with 15 mL of Aquasol-2 (New England Nuclear) for counting. A typical radioscan is shown in Figure 2b.

Preparative gas chromatography could be performed on a Varian Aerograph 90P-3 Gas Chromatograph with a 15% HIEFF-1BP on 80/100 Chromosorb WAW 6' x 1/4" outer diameter stainless steel column at a temperature of 140°, injector temperature of 147°, thermal conductivity detector temperature of 148°, collector temperature of 180° and a helium flow rate of 200 mL/min. Injecting four consecutive 0.5 uL portions of methylene chloride containing a total of 2.0 mg of cold DMN-OAc yielded a total recovery (trapped in a glass capillary tube cooled in dry ice at the outlet) of 68%.

Preparative high pressure liquid chromatography could also be applied for the purification of DMN-OAc, conditions again being worked out for the cold material. Using a Waters apparatus equipped with a semipreparative C_{18} uBondapak (Waters Associates) 7 mm by 30 cm column at room temperature with 0.05 M aqueous pH 5.5 phosphate buffer at a flow rate of 2 mL/min and an ultraviolet detector operating at wavelength 254 nm, 6.4 umole of unlabelled DMN-OAc was injected in 250 uL of buffer. Seventy-nine percent of the DMN-OAc was found by spectrophotometry in a fraction containing 18.6 mL of eluate collected so as to isolate the bulk of the major ultraviolet absorbing peak centered at 44 min. No attempt was made to further isolate the product. There were minor ultraviolet absorbing impurities at elution times of 9, 17.5, 21 and 35 min.

Stability studies. - An aqueous solution 20 mM in DMN-OAc containing enough ^{14}C to have a specific activity of 0.48 uCi/mL was subdivided into aliquots of 4 mL each. These were stored at various temperatures as listed in Table 1, and individual aliquots were removed after various durations of storage time, thawed if necessary, and analyzed by determining the absorbance at 352 nm. First order loss of absorbance was confirmed for the sample stored at +21° by following its cleanly linear decrease in ln(concentration) with time through more than one half-life; assuming first order decomposition of DMN-OAc in all solutions, the rate constants for hydrolysis at the various temperatures were calculated by

regression analysis of data sets containing four points for each line, with the results shown in Table 1.

The rate constant for loss of radioactivity from solution was followed during storage of our highest specific activity sample (62 mCi/mmole) at -18°. Aliquots of 5 uL were periodically removed after briefly thawing the frozen storage vessel, the aliquots being diluted to 10 mL with pH 5.5 phosphate buffer for scintillation counting. This result is also given in Table 1.

Labelling in the methylene group. - To test for the possibility that some of the radioactive carbon might have migrated to the methylene group of the product, a methylene chloride solution of the 0.26 mCi/mmole sample was mixed with 74 mg of cold DMN-OAc, evaporated in a stream of air, dissolved in 50 mL of pH 7.0 aqueous buffer and treated with 0.5 mg of hog liver esterase (Sigma) suspended in 2 mL of buffer. After 135 minutes, the solution was treated with 52 mL of alkaline solution containing 5 mmole of sodium hydroxide and 176 mg of 5,5-dimethyl-1,3-cyclohexane-dione (dimedone), then neutralized with a few drops of acetic acid until crystals began to form. When precipitation was complete, the white crystalline adduct was isolated by filtration and recrystallized from methanol. A 21 mg aliquot of this product was found to contain analytically significant radioactivity, corresponding to 1.3% of the total ^{14}C available.

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