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Notes

Synthesis of Radioactive Cl^{36}-Dichloromethotrexate

By VINCENT T. OLIVERIO and JACK D. DAVIDSON

THE ROLE of folic acid in cellular metabolism (1) and the mechanism of action of folic acid antagonists (2) have been the subject of numerous studies for more than a decade. While limited quantities of C^{14}-labeled folic acid have been synthesized (3, 4) for tracer studies, to our knowledge, no published reports of such studies have been carried out with radioactive labeled folic acid antagonists. The demonstration of apparent growth inhibitory properties of dichloromethotrexate[1] (DCM) in both animal tumor (5) and microbiological (6) systems offered the possibility of labeling this folic acid antagonist with radioactive chlorine. We now wish to report a successful synthesis of sufficient Cl^{36}-DCM in good yield and high purity for tracer studies.

The preparation of Cl^{36}-DCM was accomplished by a simple modification of the method described by Angier, *et al.* (7), who employed a large excess of chlorine (6.5 moles per mole of methotrexate) in order to prevent formation of monochloromethotrexate. However, to utilize the Cl^{36} from hydrochloric acid-Cl^{36} best, dimethylformamide was substituted for formamide to exclude the possible chlorination of formamide. As a result of this modification only 2.2 moles of Cl_2^{36}, obtained by hydrogen peroxide oxidation of hydrochloric acid-Cl^{36}, were required. The yield of DCM was 50% with 97% radiochemical purity.

PROCEDURE

The apparatus train consisted of a gas generator flask fitted with a dropping funnel, a reaction flask, and a sodium hydroxide gas trap connected in this order by polyethylene (PE 300) tubing. Radioactive hydrochloric acid (1.6 *N*, 7.85 ml., 250 μc.-Cl^{36}) was neutralized in the generator flask with 4 *N* sodium hydroxide. The sodium chloride-Cl^{36} was evaporated to dryness and suspended in 3 ml. of 30% hydrogen peroxide. Ten milliliters of ice-cold 15% fuming sulfuric acid, under 2.5 psi nitrogen pressure in the dropping funnel, was added dropwise to the generator flask. A slow, steady evolution of chlo-

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[1] N - [3,5 - dichloro - 4 - [(2,4 - diamino - 6 - pteridinylmethyl)methylamino]benzoyl-Cl_2^{36}}glutamic acid is the full chemical name. The abbreviated generic name, DCM, is employed throughout the text.

The hydrochloric acid-Cl^{36} was obtained from the Oak Ridge National Laboratory on allotment of the Atomic Energy Commission.

rine was maintained by the rates of addition of acid, of shaking, and of cooling. The chlorine was bubbled into a stirred (magnetic bar) ice-cold solution of 1.28 Gm. (2.82 mM) of methotrexate in 10 ml. of dimethylformamide. The effluent gas from this reaction was conducted through a trap containing 10 ml. of 1 N sodium hydroxide. Following the addition of the sulfuric acid to the gas generator the apparatus train was slowly flushed with nitrogen while the chlorine generating mixture was heated to boiling to complete the transfer of chlorine. Finally, the reaction flask was clamped off from the reaction train and stirred in the cold for one hour. In subsequent operations all mother liquors and washes were saved for radioactive assay.

The reaction mixture was poured into 100 ml. of ice-cold water and complete precipitation of crude DCM was achieved by adjusting the solution to pH 3 with 1 Gm. of sodium acetate. The precipitate was filtered and washed with water, acetone, and ether. The crude product, 1.1 Gm., was dissolved in 75 ml. of 2% sodium bicarbonate solution, heated to 75°, treated with Darco G-60 charcoal, and filtered while hot through a Celite pad on a No. 42 Whatman filter paper circle in a sintered-glass funnel. The clear yellow filtrate was reheated to 75° and approximately 2 Gm. of anhydrous magnesium sulfate added to precipitate the magnesium salt of DCM. The salt was filtered, washed with water, and dissolved in 100 ml. of water at 75°. The turbid solution was filtered hot to give a clear light-yellow colored filtrate. The warm (50°) filtrate was acidified with 1 ml. of glacial acetic acid to precipitate DCM. The pure product was filtered, washed with water, and dried at room temperature *in vacuo*. The yield was 726 mg. (49%).

The distribution of the chlorine[36] in this synthesis was determined by the liquid scintillation counting of 0.2-ml. aliquots of the various fractions, dissolved in 18 ml. of 30% methanol in toluene containing 0.3% of 2,5-diphenyloxazole and 0.01% of 1,4-di[2-(5-phenyloxazoyl)] benzene. The absolute counting efficiency was 85%. The chlorine generation, transfer, and absorption was highly efficient with only 0.08% remaining in the generator solution and less than 0.001% escaping the reaction flask to be absorbed in the alkali trap. The reaction mother liquor contained 68% of the chlorine. Another 8% appeared in the pooled water washes of the crude DCM and 4% in the acetone wash.

The pure radioactive product had a specific activity of 37 μc./mM and accounted for 20% of the chlorine[36] employed. The radiopurity of the product was assessed by paper chromatography and high voltage paper electrophoresis followed by autoradiography and counting of the radioactivity in all spots. Using ascending chromatography in sodium acetate buffer at pH 5.9, 97% of the radioactivity appeared at R_f 0.46 with 3% in a separate spot at R_f 0.12. In a descending system of 5% ammonium sulfate solution, isopropanol, and water (40:1:10), DCM had an R_f of 0.29 and a radioactive contaminant amounting to 1–2% appeared at R_f 0.36. Only a single radioactive spot appeared after high voltage electrophoresis at pH 7. At pH 3.5 the radioactive DCM migrated toward the cathode while 3% of the radioactivity moved an equal distance toward the anode and had the same mobility as authentic N^{10}-methyldichloropteroylglutamic acid. Chromatography of DCM on DEAE as previously described (8) also confirmed the 97% purity of the product. Finally, the radioactive DCM was in spectral (ultraviolet absorption) agreement with the data for DCM reported by Angier, *et al.* (7).

The 3% N^{10}-methyldichloropteroylglutamic acid contaminating our product probably arose from N^{10}-methylpteroylglutamic acid (methopterin) which is a common contaminant of methotrexate (9, 10) and was known to be present in the methotrexate employed in this synthesis. The purity of the DCM can be improved by chromatography on DEAE cellulose (8) but this appears unnecessary for studies concerned with elucidating the fate of the vast bulk, 97%, of doses of the compound. Such pharmacological studies in animals and humans are currently in progress.

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