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SHORT COMMUNICATIONS

Drug-induced porphyrin biosynthesis—VI. Uptake and biotransformation of DDC and an inactive analogue

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ON THE basis of recent studies, it appears that in order for a chemical to induce porphyria it must remain in the liver for a period of at least several hours to induce and maintain high levels of δ-aminolevulinic acid (δ-ALA) synthetase.[1] Consequently at least one of the properties that is required by a chemical in order to induce porphyria is that it must possess chemical features that prevent it from being rapidly metabolized and inactivated by the liver. In previous work with a series of esters and amides, it was noted that those esters and amides which are sterically hindered from hydrolysis by acid and base are generally active in inducing porphyria.[2] It was assumed that steric features hindering hydrolysis of these compounds by acid and base would also hinder hydrolysis of these compounds by esterases and amidases and thus prolong their sojourn in the liver. It was therefore suggested that a porphyria-inducing compound such as 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC), in which the ester functions have two neighbouring methyl groups, would be protected from hydrolysis and consequent inactivation and removal from the liver. On the other hand, the inactive analogue of DDC, viz. 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (4-desmethyl-DDC), which lacks a 4-methyl substituent should be readily hydrolyzed, inactivated and removed from the liver.

To investigate the validity of the above ideas, ^{14}C-labeled 4-desmethyl-DDC has been prepared, injected into 17-day-old chick embryos and the total radioactivity (4-desmethyl-DDC and/or metabolites) in the liver measured at different time intervals. Furthermore, we have separated 4-desmethyl-DDC from other radioactive material and measured the amount of 4-desmethyl-DDC at various time intervals. These results were compared with those obtained with DDC-^{14}C.[1]

Materials and Methods

Fertilized eggs used were of a White Leghorn strain obtained from a local hatchery and stored at 10° for no longer than 7 days prior to incubation at 38°. The age of the embryo was taken as the number of days from the onset of incubation. Ethyl acetoacetate-3-^{14}C (4·17 mc/m-mole) was purchased from New England Nuclear Corp. Melting points are uncorrected.

Instrumentation. A Bausch and Lomb Spectronic 505 was used for recording ultraviolet spectra. Nuclear magnetic resonance spectra were determined in $CDCl_3$; internal reference, tetramethylsilane; oscillator frequency, 60 × 100 cycles/sec. All radioactive samples were counted in a Nuclear Chicago model 6850 liquid scintillation system. Counts were corrected for quenching by the sample channels ratio method, and the background count was substracted. The thin-layer chromatograms containing radioactive samples were scanned using a Nuclear Chicago Actigraph 111 radiochromatography system. All radiochromatograms were scanned at a detector voltage of 960 V and gas pressure of 7 lb.

Synthesis of DDC-^{14}C. DDC-^{14}C was prepared by a method previously described.[1]

Synthesis of 4-desmethyl-DDC-^{14}*C.* This compound was prepared by a general method described by Loev and Snader[3] for the unlabelled compound. Redistilled ethyl acetoacetate (0·006 moles) was added to a vial containing ethyl acetoacetate-3-^{14}C (4·17 mc/m-mole). The contents of the vial were transferred to a 10-ml flask with 2 ml of ethanol. Formaldehyde (0·003 moles) and 0·005 moles of concentrated ammonia solution were added to the flask, and the mixture was refluxed for 3 hr. After cooling, water was added to the reaction mixture, and the product which crystallized was collected (yield 65 per cent). The product was purified by successive crystallizations from ethanol–water until the specific activity of the compound remained constant. This was achieved after three crystallizations. The product, m.p. 150–153°, was found to have a specific activity of 0·024 mc/m-mole, λ_{max} (ethanol) 231 and 375 nm (ϵ, 14,800 and 6990). Braude *et al.*[4] reported m.p. 189–190°, λ_{max} 231 and 372 nm (ϵ, 16,000 and 7250). The nuclear magnetic resonance spectrum contains a triplet at τ = 8·75 (CH_3 in ester), a singlet at τ = 7·83 (2 and 6 CH_3 on ring), a singlet at τ = 7·12 (not assigned)

a singlet at $\tau = 6{\cdot}75$ (H on ring), a quartet at $\tau = 5{\cdot}85$ (CH_2 in ester) and a broad band at $\tau = 4{\cdot}7$ (H on nitrogen). The relative peak areas were 6·5:6·0:1·3:1·8:4·1:0·6. Schroll *et al.*[5] reported similar nuclear magnetic resonance data but did not report a singlet at $\tau = 7{\cdot}12$.

Synthesis of 3,5-*diethoxycarbonyl-2,6-dimethylpyridine-*^{14}C (*4-desmethyl-Ox-DDC*). This compound was prepared from 4-desmethyl-DDC-^{14}C by a general method described by Braude *et al.*[4] for the unlabeled compound. The product was purified by two crystallizations from ethanol–water at which point the specific activity of the compound remained constant. The product, m.p. 70–71°, was found to have a specific activity of 0·024 mc/m-mole, λ_{max} (ethanol) 235, 273 and 281 nm (ϵ, 11,500, 3930 and 3260). Braude *et al.*[4] reported m.p. 73·5–74·5°, λ_{max} 236 and 282 nm (ϵ, 13,200, 4120 and 3380). The nuclear magnetic resonance spectrum contains a triplet at $\tau = 8{\cdot}58$, (CH_3 in ester), a singlet at $\tau = 8{\cdot}58$, (CH_3 in ester), a singlet at $\tau = 7{\cdot}14$ (CH_3 on ring), a quartet at $\tau = 5{\cdot}58$ (CH_2 in ester) and a singlet at $\tau = 1{\cdot}29$ (H on ring). The relative peak areas were 5·7:6·0:3·7:0·9.

Amount of ^{14}C *and of unchanged drug in livers at different time intervals after injection of* ^{14}C*-labeled drugs into the fluids surrounding the chick embryo.* An accurately weighed amount (4 mg) of labelled drug was dissolved in 0·1 ml of dimethylsulfoxide and injected into the fluids surrounding the 17-day-old chick embryo. After various periods of incubation, the livers of the embryos were removed and the total radioactivity (unchanged drug and/or metabolites) in the liver determined by liquid scintillation counting.[1]

In order to determine the amount of unchanged drug in the livers at various time intervals after the injection of 4 mg of labeled drug into fluids surrounding the embryo, the livers were removed, and the drug and its metabolites were extracted with methanol. The drug and its metabolites were separated by thin-layer chromatography and the amount of each fraction determined by liquid scintillation counting.[1]

Results and discussion

The first objective of this study was to synthesize, 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C in radiochemically pure form. The synthesis of the unlabeled compound yielded a product with m.p. 158–163°. Since the melting point of our compound differed from that reported by Braude *et al.*,[4] namely 189–190°, alternate routes of synthesis were explored but the product obtained was not superior to that obtained by the previous procedure. It appeared possible that the melting point was not a good criterion of purity and that ready oxidation to the corresponding pyridine might explain the low melting point observed. It was decided to assess the purity of 4-desmethyl-DDC as follows. 4-Desmethyl-DDC was crystallized to constant specific activity (0·024 mc/m-mole) and oxidized to 3,5-diethoxycarbonyl-2,6-dimethylpyridine whose physical properties, described in the

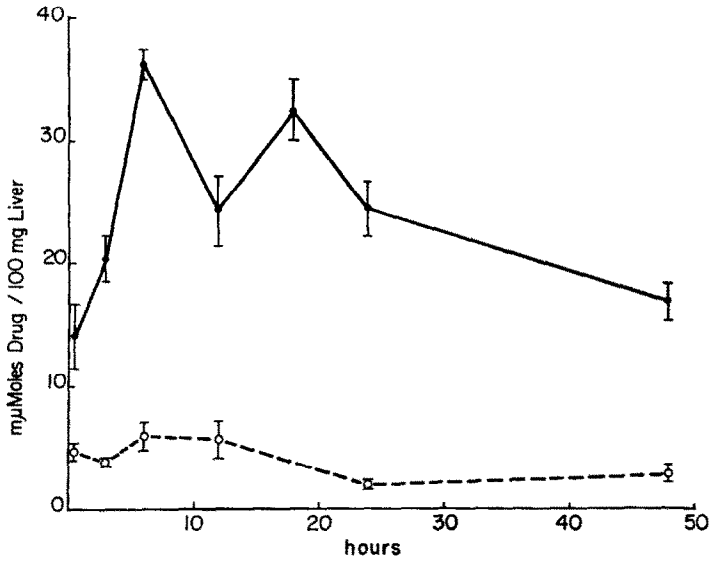

FIG. 1. Amount of radioactive drug (unchanged drug and/or metabolites) in livers of 17-day-old chick embryos at different time intervals after injection of DDC-^{14}C (●——●) and 4-desmethyl-DDC-^{14}C (○- - -○). Each point represents mean ± S. E. of mean.

experimental section, were the same as those previously reported by Braude *et al.*[4] Since the specific activity of the pure pyridine (found to be 0·024 mc/m-mole) was the same as that of the dihydropyridine, it was clear, that if the dihydropyridine contained an impurity, it was present in very small quantities.

The second objective of this study was to measure the total amount of radioactivity and unchanged drug in the livers of 17-day-old chick embryos at different time intervals after the injection of 4-desmethyl-DDC-^{14}C. The total amount of radioactive drug in the livers of chick embryos at different time intervals was compared to results previously obtained with DDC-^{14}C, and summarized in FIG. 1. The amount of radioactivity (representing 4-desmethyl-DDC and/or metabolites) present in the liver at any specific period in time after injection of 4-desmethyl-DDC was approximately one-fifth of the amount after injection of an equimolar amount of DDC-^{14}C. This observation was in accordance with the expectation that 4-desmethyl-DDC would be more readily metabolized and therefore not achieve as high a concentration in the liver as DDC.

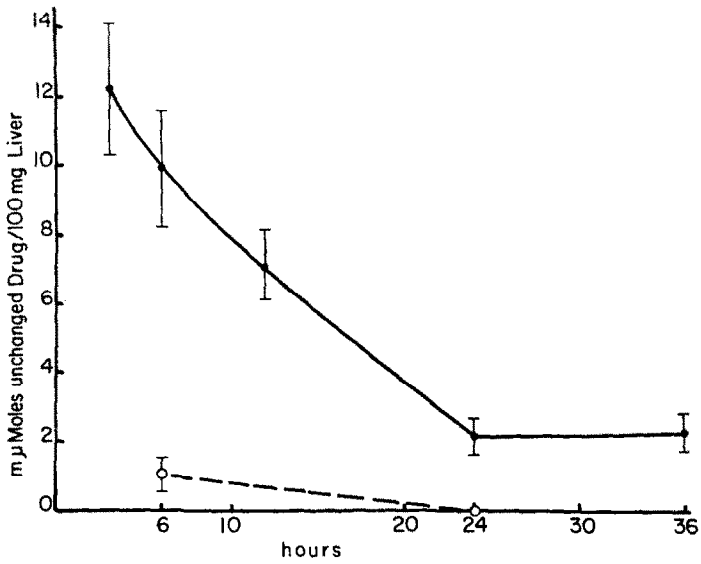

FIG. 2. Amount of unchanged drug in livers of 17-day-old chick embryos at different time intervals after injection of DDC-^{14}C (●——●) and 4-desmethyl-DDC-^{14}C (○– – –○). Each point represents mean ± S. E. of mean.

The total radioactivity (representing 4-desmethyl-DDC and/or metabolites) in the livers of chick embryos was extracted at various time intervals after injection and separated by means of thin-layer chromatography using benzene–methanol (14:1) as the developing solvent. The plates were scanned by means of a radiochromatogram scanner, and areas of Silica gel containing radioactivity were removed and counted. It was observed that 4-desmethyl-DDC was oxidized to the corresponding pyridine during the extraction procedure, and it was therefore assumed that the 4-desmethyl-Ox-DDC recovered from the thin-layer plates represented 4-desmethyl-DDC which had been oxidized in the course of the extraction procedure. Therefore the total unchanged drug in the liver (FIG. 2) was calculated by adding the amounts of 4-desmethyl-Ox-DDC and 4-desmethyl-DDC obtained from the thin-layer plate. Therefore unchanged drug represents 4-desmethyl-DDC and its oxidation product but excludes metabolic products. The amount of unchanged drug at different time periods after injection was compared (FIG. 2) with results previously obtained with DDC.[1] Clearly, there is far less unchanged 4-desmethyl-DDC in the liver than unchanged DDC. This observation is in accordance with the expectation that 4-desmethyl-DDC would be more readily metabolized and therefore not achieve as high a concentration in the liver as DDC. Further studies are in progress to delineate the details of the metabolism of DDC and its analogues.

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Effect of different routes of administration of cedrene on hepatic drug metabolism

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WHEN mice or rats are exposed to cedarwood bedding, they exhibit an increase in the metabolism of certain drugs.[1–3] Wade *et al.*[3] showed that this increase was due to the induction of hepatic microsomal enzymes by cedrene, the major constituent of cedarwood oil, and suggested that the main route of administration was by inhalation; however, they provided no estimate of the dose of cedrene required for induction. The present investigation was undertaken to estimate the dose of cedrene absorbed via inhalation and to compare the effect of different routes of administration of cedrene on hepatic drug metabolism.

Female, Sprague–Dawley rats (150–200 g) were obtained from Hormone Assay (Chicago, Ill.). Various doses of cedrene in corn oil (1 ml/kg) were injected orally or intraperitoneally once a day for 3 days. Control animals received corn oil (1 ml/kg). Rats were sacrificed 24 hr after the last oral or intraperitoneal administration of cedrene. When cedrene was administered to rats by inhalation, the rats were maintained in the inhalation chamber continuously except for 1 hr a day during which time the chamber was cleaned.

The system employed for the inhalation studies consisted of a drying tower ($CaCl_2$), an air flow meter, two gas washing bottles containing the cedrene, an inflow air reservoir, an inhalation chamber, and an outflow air reservoir (Fig. 1). A standard 15-l. desiccator was used for the inhalation chamber,

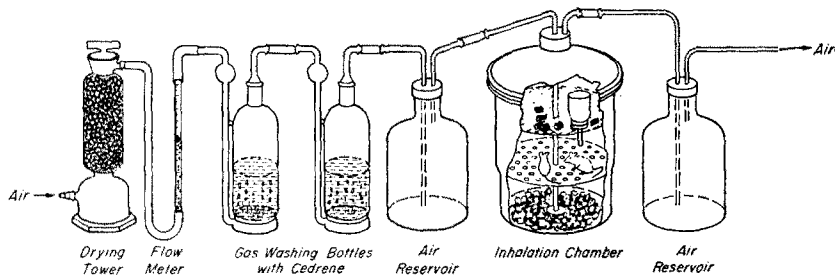

FIG. 1. Schematic diagram of inhalation system.