Impurities in Labeled Diethylstilbestrol: Identification of Pseudodiethylstilbestrol

Abstract. *Radioactive impurities known to exist in labeled diethylstilbestrol used in tissue residue studies in cattle include at least five different compounds, one of which is identified as pseudodiethylstilbestrol.*

Investigators at the U.S. Department of Agriculture (USDA) laboratories in Fargo, North Dakota, and in Beltsville, Maryland, have reported (*1–4*) the results of studies in cattle given radioactively labeled diethylstilbestrol (DES) both orally and by implantation. In one of these studies (*2*) it was determined by isotope dilution that 5 percent or more of the total radioactivity recovered from the implants used was in the form of labeled impurities. As the daily absorption of these impurities is sufficient to account many times over for the total radioactivity content in the beef livers, and as most of the radioactivity in the livers was not in the form of DES or DES conjugates (*4*), the presence of labeled impurities in the DES formulation used is a matter of concern. The likelihood that the same impurities may follow nonradioactive carriers added during isolation procedures confounds residue identification by the isotope dilution method.

We report here the isolation and characterization of the principal radioactive impurity in the formulated material used in one of the USDA studies with DES implants (*4*). The labeled DES contained ^{14}C in the terminal position of one ethyl group. In April 1972 the supplier, Amersham/Searle, reported the specific activity as 58.4 curie/mole (93 percent labeled) and the radiochemical purity as 99 percent, determined by one-dimensional thin-layer chromatography, and 98 percent, determined by paper chromatography. The labeled DES was mixed with hydrogenated peanut oil and calcium stearate at Hess and Clark and compressed into the experimental implants used by the USDA. There remained a small amount of uncompressed formulation, which was taken up in benzene while the cattle tests were in progress. The studies reported here were conducted with a part of this solution.

Qualitative examination consisted of two-dimensional chromatography on silica gel thin-layer plates with methylene chloride as the solvent in the first direction and a mixture of benzene and ether (95 : 5) as the solvent in the second direction. Radioautograms were prepared by exposing x-ray film to the developed chromatograms for periods of 2 to 5 days.

Table 1 presents the results. Very prominent zones for *cis*- and *trans*-DES exhibited characteristic yellow fluorescence on the chromatographic plate on exposure to ultraviolet light. Seven other radioactive areas could be discerned, including one at the origin. To determine which zones might be artifacts, we removed the adsorbent from the area of the *trans*-DES zone of a plate, eluted the DES with ether, applied it to another plate, and developed again. Zones corresponding to *cis*- and *trans*-DES, origin, and zone 6 appeared.

Table 1 also presents the mean relative amounts of radioactivity associated with the several zones in six replicate separations, determined by suspending the silica gel from the different areas in diotol and counting by liquid scintillation. More than 5 percent of the radioactivity was not associated with DES.

We have identified the most prominent zone, Zone 4, by mass spectrometry. The material was isolated from several chromatographic plates and purified by rechromatography on a plate that had been previously washed with redistilled developing solvents. The final silica gel adsorbate was packed in a microcolumn and successively washed with two 1-ml portions of ethanol. The first eluate, containing about 380 ng of radioactive compound, was taken to dryness and approximately one-half of it was subjected to direct-probe mass spectrometry. Figure 1 presents the spectra for the unknown compound and for unlabeled diethylstilbestrol. The spectrum of DES is characterized by a molecular ion (base peak) of mass-to-charge ratio (*m/e*) 268, with fragment ions of *m/e* 253 (loss of methyl; 14 percent of base peak) and *m/e* 239 (loss of ethyl; 57 percent). The unknown gave intense signals of *m/e* 270 [only a weak (approximately 5 percent) signal of the same intensity as neighboring background peaks was observed at 268], 239, and 241, and a pair of low-intensity ions of *m/e* 253 and 255. Ion *m/e* 270 is the molecular ion of a compound with the same elemental composition as DES but containing one ^{14}C atom per molecule, the situation that should pertain with specific activity of ~ 60 curie/mole. The doublets resulting from losses of both 29 and 31 atomic mass units (amu) ([^{12}C]- and [^{14}C]ethyl) and 15 and 17 amu ([^{12}C]- and [^{14}C]methyl) are compelling evidence that the spectrum arises from a compound related to [^{14}C]DES labeled in the ethyl group, specifically the methyl carbon. The intensities of the signals at *m/e* 239 and 241 (88 and 85 percent, respectively) relative to the molecular ion are, however, much greater than the intensity of the *m/e* 239 signal in the spectrum of reference DES. The unknown also shows an intense triplet of signals at *m/e* 133, 135 (base peak), and

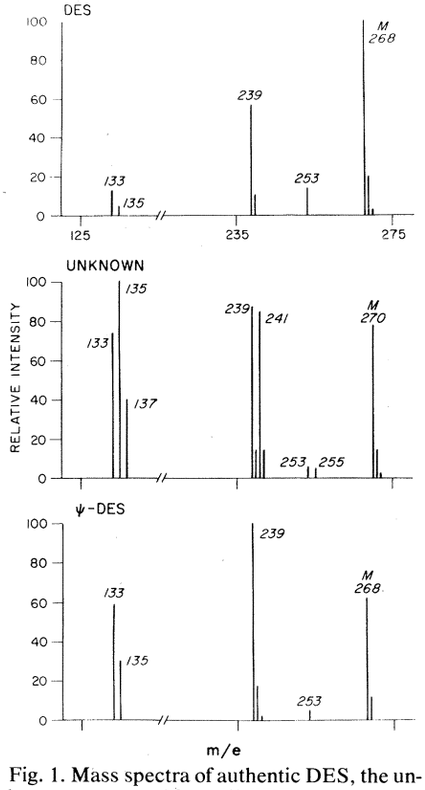


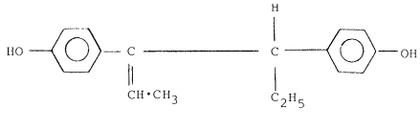
Fig. 1. Mass spectra of authentic DES, the unknown compound from [^{14}C]DES, and authentic pseudo-DES (ψ-DES) obtained by using the following instrument settings: ionizing potential, 70 ev; trap current, 60 μa; accelerating potential, 3.5 kv; and source temperature, 250°C.

Table 1. R_F values and ^{14}C content of radioactive zones in two-dimensional thin-layer chromatograms of DES implant formulation.

Zone	R_F values		^{14}C (%)
	First direction	Second direction	
Origin	0	0	2.3
cis-DES	0.20	0.12	23.2
trans-DES	0.49	0.30	68.5
1	0	0.32	0.7
2	0.03	0.02	0.9
3	0.10	0.22	0.5
4	0.25	0.16	2.1
5	0.50	0	1.4
6	0.85	0.54	0.4

137, whereas DES gave only low-intensity signals at *m/e* 133 and 135 and no signal at *m/e* 137.

The residue from the second ethanol solution, containing about 190 ng of the unknown, was dissolved in 5 μl of bis(trimethylsilyl)acetamide, and one-half was examined by mass spectrometry after a reaction time of 2 minutes. The spectrum exhibited signals at the *m/e* values required by conversion of the compound to a di-trimethylsilyl derivative: 270 → 414; 253/255 → 397/399; 239/241 → 383/385; and 133/135/137 → 205/207/209. The appropriate shifts also occurred after trimethylsilylation of DES. These data suggest that the unknown is an isomer of DES, and one possibility is pseudo-DES.



This compound should display an intense signal for loss of an ethyl group, as the resulting ion with *m/e* 239 is both benzylic and allylic. Scission of the C-C bond between the two benzyl carbons results in the formation of ions of *m/e* 133 ($^{12}C_9H_9O$), 135 ($^{12}C_9H_{11}O$ and $^{12}C_8{}^{14}CH_9O$), and 137 ($^{12}C_8{}^{14}CH_{11}O$). Analogous ions for the trimethylsilyl derivative will appear at *m/e* 205, 207 and 209. The spectra obtained for an authentic sample of pseudo-DES (*5*) (free and derivative) match those for the unknown when the ^{14}C atom in the latter is taken into account (see Fig. 1), and the two substances behave identically in thin-layer chromatography.

Radiochemical purity is of transcending importance in tissue residue studies, particularly in view of current emphasis on analytical sensitivities and identifications at picogram levels. We have identified only one of several impurities present in the isotopic DES implants, and this was not apparent in the routine analytical procedure used for initial reporting of radiochemical purity. The labeled impurities found here may arise not only from the initial radioactive synthesis, but also from self-radiolysis (*6*) in the solid state during storage and during the experimental period.

D. M. TENNENT
R. F. KOUBA, W. H. RAY
Rhodia Inc., Hess and Clark Division, Ashland, Ohio 44805
W. J. A. VANDENHEUVEL
F. J. WOLF
Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey

References and Notes

1. P. W. Aschbacher and E. J. Thacker, *J. Anim. Sci.* **39**, 1185 (1974).
2. P. W. Aschbacher, E. J. Thacker, T. S. Rumsey, *ibid.* **40**, 530 (1975).
3. T. S. Rumsey, R. R. Oltjen, F. L. Daniels, A. S. Kozak, *ibid.*, p. 539.
4. T. S. Rumsey, R. R. Oltjen, A. S. Kozak, F. L. Daniels, P. W. Aschbacher, *ibid.*, p. 550.
5. The *cis* isomer was obtained from Chemetron Corporation.
6. P. Rochlin, *Chem. Rev.* **65**, 685 (1965).

15 June 1976; revised 8 September 1976

Choline Administration: Activation of Tyrosine Hydroxylase in Dopaminergic Neurons of Rat Brain

Abstract. *The administration of choline in doses previously shown to elevate brain acetylcholine concentrations also increases the activity of tyrosine hydroxylase in rat caudate nuclei. This response can be blocked by atropine, a muscarinic antagonist. These findings indicate that choline-induced increases in acetylcholine concentrations may be associated with parallel changes in the amount of the neurotransmitter released into synapses.*

The administration of choline by injection (*1*) or diet (*2*) raises the concentration of the neurotransmitter acetylcholine (ACh) in rat brain. This increase may or may not be associated with a change in the amount of transmitter actually released into synapses per unit time. Studies described in this report show that, by activating central muscarinic receptors, choline administration also elevates the activity in caudate nuclei of tyrosine hydroxylase (TOH), the enzyme that catalyzes the first step in catecholamine biosynthesis. This enzyme is absent from cholinergic neurons but is present in dopaminergic neurons that receive cholinergic inputs (*3*). Hence, its activation by choline suggests that choline administration actually does enhance ACh release, at least within the brain.

Male Sprague-Dawley rats (Charles River) weighing 150 to 200 g were housed in groups of eight in a controlled environment (23° to 24°C) for 2 to 4 days before use in an experiment. Animals had free access to food and water and were exposed to light (Vita-Lite, Duro-Test Corp., North Bergen, N.J.) daily between 7 a.m. and 7 p.m. Choline chloride (ChCl) was dissolved in saline and injected intraperitoneally in a total volume of 2 ml per kilogram of body weight. Rats were decapitated 2 hours after injection, and their brains were quickly removed. The corpora striata were dissected (*4*) on an ice-cooled glass plate and immediately frozen on Dry Ice. Frozen tissues were weighed and homogenized in 10 volumes of 50 m*M* tris-acetate buffer, *p*H 6, containing 0.2 percent Triton X-100. The homogenates were placed in an ice bath for 20 to 30 minutes and were then centrifuged at 10,000*g* for 10 minutes. The TOH activity in the supernatant fluid was assayed by the method of Waymire *et al.* (*5*) and the protein concentration by the method of Lowry *et al.* (*6*).

A single injection of 60 or 120 mg of ChCl per kilogram caused striatal TOH activity to increase by 19 percent ($P < .05$) or 36 percent ($P < .01$), respectively (Table 1). A smaller dose (30 mg/kg) failed to elevate TOH activity significantly. We have previously shown that this lower dose causes peak increments of 11 percent in brain ACh levels, while the 60-mg/kg dose causes brain ACh to rise by 22 percent (*1*). [We were unable to measure ACh and TOH activity in the same tissue samples because measurement of ACh requires that brain enzymes be inactivated—for example, by a focused microwave beam aimed at the head (*1, 2*).] The increase in TOH activity that follows choline administration could be blocked by treating rats concurrently with atropine sulfate, 40 mg/kg, intraperitoneally (Table 2). This muscarinic antagonist had no effect on TOH activity when injected alone (Table 2).

Javoy *et al.* (*7*) have recently shown that dopa synthesis (*8*) in the corpus striatum is accelerated when rats are treated with oxotremorine, a drug that stimulates central muscarinic receptors. In preliminary studies, we noted a similar acceleration of dopa synthesis in rats given choline (*9*). In animals killed 30 minutes after receiving RO-4-4602 (800 mg/kg, intraperitoneally), those that had also received choline 90 minutes before death had striatal dopa concentrations (957 ± 84 ng/g) 42 percent higher than those of rats that received only the dopa decarboxylase inhibitor (674 ± 45 ng/g, $P < .01$).

Choline itself reportedly acts as a very weak agonist on muscarinic receptors; however, the brain choline levels attained after animals received the choline doses used here (*1*) were probably far too low for the choline to have any direct effect on ACh receptors. Hence, the activation of these receptors after choline administration most likely resulted from in-