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Observations on the Nature of Radioactive Contaminants in Biosynthetic Cholesterol-C^{14}.* (20728)

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Schwenk and co-workers have reported that the digitonide of biosynthetic cholesterol-C^{14} is contaminated in certain instances by unidentified compounds of high specific radioactivity(1-3). This phenomenon has been of particular interest because of the possibility that the contaminating substances might represent biological precursors of cholesterol.

It has been found in this laboratory that up to 80% of the radioactivity of the sterol digitonide obtained from the livers of rats sacrificed 3 minutes after injection of sodium acetate-1-C^{14} was due to non-cholesterol contaminants. A number of substances related either chemically or biologically to cholesterol have been examined as possible sources of radioactive contamination.

Experimental. Each experiment employed cholesterol digitonide obtained from the pooled livers of several animals. Normal adult male Wistar rats were anesthetized by intraperitoneal injection of sodium pentobarbital, 6 mg per 100 g body weight. After exposure of the femoral vein, each animal was given 4.0-6.7 mg of sodium acetate-1-C^{14} intravenously (total radioactivity 5.8-13.4 x 10^6 counts/min). Three minutes later the liver was rapidly excised and immersed into 10 volumes of hot 95% ethanol containing 12% KOH. The mixture was boiled for 3 hours under reflux and filtered through sintered glass. Sufficient water was added to the filtrate to reduce the ethanol concentration to 50%, and the non-saponifiable material was obtained by 3 extractions with one-half volume of petroleum ether (B.P. 60-68°). The petroleum ether extracts were combined, washed once with 5% sodium acetate and twice with water, then dried over Na_2SO_4 and evaporated to dryness under N_2. The residue was taken up in 1:1 acetone-ethanol, and cholesterol digitonide was precipitated by the addition of a 1% solution of digitonin in 80% ethanol. The digitonide was washed twice with 80% ethanol and once with ether.

In a preliminary experiment sodium acetate-1-C^{14} containing 2.5 x 10^5 counts/min was added to the liver of a normal rat, and cholesterol digitonide isolated as described above. The digitonide was free of radioactivity. Radioactivity assay was carried out with a windowless flow gas counter. Cholesterol, 3β-cholestanol and 7-ketocholesterol were counted as the digitonides, mounted on sintered alundum discs. Other compounds were subjected to wet combustion by a modification of the method of Lindenbaum *et al.*(4) and counted as $BaCO_3$ mounted on alundum discs.

Exp. 1. The digitonide obtained in one ex-

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periment had a specific radioactivity of 39 counts/min/μmole. The free sterol was prepared from this digitonide with pyridine and ether. Cholesterol isolated via the dibromide (1) from a portion of this crude sterol had a specific activity of only 12 counts/min/μmole. 5.7 mg of unlabeled 7-ketocholesterol and 9.0 mg of unlabeled 7β-hydroxycholesterol were added to 8.5 mg of the crude sterol. This mixture was separated by column partition chromatography, using the solvent system propylene glycol-petroleum ether(5). The digitonide prepared from the cholesterol band had a specific activity of 33 counts/min/μmole; both the 7-ketocholesterol and 7β-hydroxycholesterol bands were non-radioactive within the precision of measurement. Another portion (6.5 mg) of the crude sterol was chromatographed with 7.9 mg of cholestane-3β,5α,6β-triol on a partition column using the solvent system aqueous methanol-cyclohexane(5). The digitonide prepared from the cholesterol band had 34 counts/min/μmole, while the triol was non-radioactive. A mixture of 3 mg of unlabeled cholesterol and 0.5 mg of acetic acid-1-C^{14}, containing 1 x 10^6 counts/min, was chromatographed using the system propylene glycol-petroleum ether. The eluted cholesterol was free of radioactivity.

Exp. 2. Another sample of "contaminated" cholesterol digitonide had a specific activity of 163 counts/min/μmole. 50 mg of free sterol obtained from this digitonide were brominated; the 5,6-dibromocholestan-3β-ol had a specific activity of 35 counts/min/μmole. After debromination, the specific activity was 33 counts/min/μmole. To the mother liquor from the bromination were added 2.45 mg of non-radioactive 3β-cholestanol, and the digitonide of the saturated sterol fraction was precipitated. 11.9 mg of digitonide with a specific activity of 298 counts/min/μmole were obtained. It was calculated that all but 1.7 mg of this was inactive carrier digitonide, and that the activity of carrier-free sterol was approximately 2,100 counts/min/μmole. After adding unlabeled 3β-cholestanol digitonide as additional carrier, the free sterol was regenerated and isolated. The specific activity was 16 counts/min/μmole, a value consistent with the dilution by carrier. After 3 crystallizations from methanol, the specific activity fell to 1.4 counts/min/μmole. After 9 recrystallizations the 3β-cholestanol was free of detectable radioactivity.

Exp. 3. Another portion of the "contaminated" cholesterol with specific radioactivity of 163 counts/min/μmole was subjected to reversed-phase partition chromatography(6) employing heptane as the stationary phase and aqueous methanol as the mobile phase. Elutions were made with 90, 92.5, 95 and 100% methanol. Cholesterol was eluted with 92.5% methanol; the digitonide prepared from this fraction had an activity of 93 counts/min/μmole. The remainder of the radioactivity was found in fractions of negligible weight appearing just before and just after the cholesterol band. With this solvent system, squalene was eluted with 100% methanol; this fraction was free of radioactivity.

Summary. Experiments have been carried out on the nature of the radioactive contaminants in biosynthetic cholesterol-C^{14} obtained from rat livers, employing preparations in which as much as 80% of the radioactivity was due to contaminating material. At least one major contaminating substance was still precipitable with digitonin after treatment with bromine, but it was not 3β-cholestanol. The data make it unlikely that 7-ketocholesterol, 7β-hydroxycholesterol, cholestane-3β,5α,6β-triol, squalene or unutilized acetate contributed significantly to the radioactive contamination found in these preparations.

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