THE ISOLATION OF CRYSTALLINE 22R-HYDROXYCHOLESTEROL AND 20α,22R-DIHYDROXYCHOLESTEROL FROM BOVINE ADRENALS.

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SUMMARY: Using tritiated internal standards, crystalline 22R-hydroxycholesterol and 20α,22R-dihydroxycholesterol have been isolated for the first time from bovine adrenal glands. The identity of the sterols was established by comparison of their melting points and infrared spectra with those of authentic standards. The tissue concentration of the sterols, as determined by both direct weighing of crystalline samples and the double-isotope derivative technique, indicated the presence of 1.5 mg/kg of 22R-hydroxycholesterol and 2.15 mg/kg of 20α,22R-dihydroxycholesterol. The presence of neither the monosulfate nor the disulfate of 22R-hydroxycholesterol could be established.

The biosynthetic pathway by which pregnenolone is produced in adrenal glands from cholesterol reportedly involves three hydroxylated intermediates; 20α-hydroxycholesterol (cholest-5-ene-3β,20α-diol), 22R-hydroxycholesterol (cholest-5-ene-3β,22R-diol) and 20α,22R-dihydroxycholesterol (cholest-5-ene-3β,20α,22R-triol). However, the evidence for the existence of these intermediates has been based primarily on incubation studies in which radioactive tracers of these compounds were converted into pregnenolone. Until now, however, none of these hydroxylated sterols has been isolated from endocrine tissues in an acceptably characterized form. Recently, Burstein *et al.* (1) demonstrated the conversion of tracer amounts of radioactive cholesterol into 22R-hydroxycholesterol and 20α,22R-dihydroxycholesterol in adrenal preparations, without using internal trapping agents as others had before (2-4). The identities of the radioactive products were established by acceptable means. The present data conclusively prove that 22R-hydroxycholesterol and 20α,22R-dihydroxycholesterol are naturally-occurring constituents of adrenal tissue.

Isolation of 22R-hydroxycholesterol. 5 kg of bovine adrenal glands were extracted with methanol containing 1.08 x 10^6 cpm of 22R-hydroxy-22-^{3}H-cholesterol* (specific activity 223,000 cpm/μg) using a procedure described by Roberts *et al.* (5). Concentration of the methanolic extract *in vacuo* resulted in an aqueous solution which was extracted with Skellysolve B. Removal of the solvent from the organic extract left an oil which weighed 8 g and contained 483,000 cpm. The residue was chromatographed on 100 g of alumina containing 6% water and yielded a fraction, eluted with Skellysolve B:benzene (1:1), which weighed 0.43 g and contained 442,000 cpm. The material was rechromatographed on 60 g of alumina and the ^{3}H-labeled material was eluted with 30% ether in benzene. The residue (15 mg) was rechromatographed on 66 g of Celite (6) using isooctane:methanol:water (10:9:1). A single, symmetrical peak of a radioactive product containing 381,000 cpm was eluted in hold-back volume (HBV) 4. Solvent removal afforded an almost colorless residue which was crystallized from Skellysolve A to give needles melting at 184.5-185°. Authentic 22R-hydroxycholesterol, when crystallized from ether, melts at 184-185°. The infrared spectrum of the isolated material in KBr was identical with that of 22R-hydroxycholesterol. The specific activity of the isolated crystalline material, as determined by direct weighing, was found to be 154 cpm/μg. Acetylation with 1-^{14}C-acetic anhydride of known specific activity [desoxycorticosterone-^{14}C-acetate (DOCA) prepared with a sample of the radioactive anhydride has a specific activity of 17.5 cpm/μg] and crystallization of the diacetate from aqueous methanol yielded plates which melted at 103-104°. Authentic 22R-hydroxycholesterol diacetate melts at 102-104°. The ^{3}H/^{14}C ratios of these crystals and of its mother liquor were 4.96 and 4.62, respectively. Following dilution with 10 mg of carrier 22R-hydroxycholesterol diacetate and two successive crystallizations from aqueous methanol, the ^{3}H/^{14}C ratios were found to be:

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* 22R-hydroxy-22-^{3}H-cholesterol was prepared by reduction of 22-ketocholesterol with lithium aluminum tritide in tetrahydrofuran.

1st crystals 4.92; mother liquor 4.89; 2nd crystals 4.86. Based on the $^{3}H/^{14}C$ ratios, the specific activity of the isolated material was calculated to be 161 cpm/μg (6). From the average of the two specific activities (157 cpm/μg), one obtained by direct weighing (154 cpm/μg) and the other by the double-isotope derivative technique (161 cpm/μg), the amount of 22R-hydroxycholesterol present in the original batch of bovine adrenals was estimated to be 1.5 mg/kg.

Isolation of 20α,22R-dihydroxycholesterol. Employing 1 kg of bovine adrenal tissue and 200,000 cpm of 20α,22R-dihydroxy-22-^{3}H-cholesterol*, a methanolic extract was prepared as previously described. The extract was defatted by reversed-phase partition column chromatography on 1 kg of Celite using methanol:n-propanol:water:toluene:isooctane (4:1:1:3:2:2) as described by Roberts *et al.* (5) and rechromatographed on 500 g of Celite with isooctane:methanol:water (10:8:2). The tritiated internal standard was eluted in HBV 7 and solvent evaporation yielded a residue which weighed 70 mg and contained 134,000 cpm. Further chromatography on 100 g of Celite using toluene saturated with propylene glycol reduced the radioactive fraction to an oil which weighed 12 mg. The residue was rechromatographed on a thin layer of silica gel with chloroform:ether (1:1) against a marker of 20α,22R-dihydroxycholesterol and the area of the plate corresponding to the marker was eluted with acetone. Evaporation of the solvent yielded a colorless residue which weighed 2.6 mg and contained 97,000 cpm. Crystallization from acetone:Skellysolve A gave plates which melted at 141-154°. An authentic sample of 20α,22R-dihydroxycholesterol melts unsharply between 155-172°. The infrared spectrum (KBr) of the isolated material and that of the authentic standard were identical. Acetylation with 1-^{14}C acetic anhydride (specific activity of DOCA pre-

* 20α,22R-dihydroxy-22-^{3}H-cholesterol was prepared enzymatically by incubation of 22R-hydroxy-22-^{3}H-cholesterol with a bovine adrenal mitochondrial acetone powder. One radioactive metabolite, more polar than the starting material, was obtained. By virtue of its chromatographic behavior, it was assumed to be the 20α,22R-glycol and this was later confirmed when this radioactive product was found to be identical with the crystalline glycol isolated from the adrenal extract.

pared with this sample was 16.4 cpm/μg) and crystallization from methanol gave needles which melted at 178-180°. A similarly-prepared sample of authentic 20α,22R-dihydroxycholesterol-3,22-diacetate melts at 178-182°. The doubly-labeled acetate was crystallized twice from methanol to give the following $^{3}H/^{14}C$ ratios: 1st crystals 3.19; 1st mother liquor 2.68; 2nd crystals 3.13; 2nd mother liquor 3.08. The specific activity of the isolated 20α,22R-dihydroxycholesterol was calculated to be 93 cpm/μg from which its concentration in bovine adrenal tissue was estimated to be 2.15 mg/kg.

A search for 22R-hydroxycholesterol 3-monosulfate and 22R-hydroxycholesterol 3,22-disulfate. 110 g of bovine adrenal tissue was homogenized in 1 liter of 0.5% aqueous sodium bicarbonate containing 340,000 cpm of the sodium salt of 22R-hydroxy-22-^{3}H-cholesterol 3-monosulfate* (specific activity 54,000 cpm/μg) and 191,000 cpm of the disodium salt of 22R-hydroxy-22-^{3}H-cholesterol 3,22 disulfate. Following the removal of the solids, the solution was saturated with NaCl and extracted with 2 volumes of tetrahydrofuran (THF). The residue from the THF extract was chromatographed on 130 g of Celite using isooctane:ethyl acetate:t-butanol:methanol:1% aqueous $NaHCO_3$ (3:3:2:2:3). The monosulfate fraction (1.1 mg) was eluted in HBV 7 and contained 240,000 cpm. It was solvolyzed in THF with $HClO_4$ (8) and the product, after chromatography on Celite, was acetylated with 1-^{14}C-acetic anhydride. The diacetate was diluted with carrier 22R-hydroxycholesterol diacetate and recrystallized to a constant $^{3}H/^{14}C$ ratio. The ratio was found to be identical with that of the internal standard. The disulfate was eluted from Celite with methanol. It was chromatographed on Celite using isooctane:ethyl acetate:t-butanol:methanol:1% aqueous NaHCO (1:4:2:1:3), solvolyzed and acetylated with 1-^{14}C-acetic

* 22R-hydroxy-22-^{3}H-cholesterol 3-monosulfate was prepared by reduction of 22-ketocholesterol 3-monosulfate (triethylammonium salt) with sodium borotritide. The C-22 hydroxy epimers were separated by partition column chromatography and the specific activity of the 22R-epimer determined by acetylation with 1-^{14}C-acetic anhydride following solvolysis. 22R-hydroxy-22-^{3}H-cholesterol 3,22-disulfate was prepared by reaction of 22R-hydroxy-22-^{3}H-cholesterol with triethylamine sulfur trioxide in pyridine (7). The product was homogeneous upon partition chromatography and did not form an acetate.

anhydride. The $^{3}H/^{14}C$ ratio in the diacetate was identical with that of the internal standard.

Thus, it would appear that neither 22R-hydroxycholesterol 3-monosulfate nor 22R-hydroxycholesterol-3,22-disulfate is present in bovine adrenal tissue.

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