Enteric Excretion of Metabolites of Steroid Hormones in the Human. III. Isolation of 3α-Hydroxy-5β-pregn-16-en-20-one (16-Pregnenolone) from Meconium*

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3α-Hydroxy-5β-pregn-16-en-20-one (16-pregnenolone) was isolated from meconium excreted by full-term male and female infants during the first 24 hours after birth. It was obtained after hydrolysis by bacterial β-glucosiduronidase and was present in a concentration of about 1.3 mg per kg of meconium. The isolation of this steroid by mild methods of hydrolysis, extraction, and purification and the finding of an amount of the steroid in meconium which is significant when compared with the concentrations of the other steroids isolated from this source suggest that 16-pregnenolone may be a true metabolite. Although the importance of 16-pregnenolone in the endocrine metabolism of the fetus has not been evaluated, it is suggested that this steroid may be an intermediate in an alternate route by means of which 17-deoxy C-21 compounds may be transformed to C-19 and C-18 steroids with oxygen functions at carbons 16 and 17.

The investigation of meconium, the contents of the intestinal tract of the fetus and the newborn infant, offers a unique and reasonably convenient means of examining the endocrinologic relationships which exist between the human maternal organism, the placenta, and the fetus. Studies of meconium as a source of metabolites of steroid hormones have resulted in the isolation of estriol (Kinsella *et al.*, 1956), estriol-glucosiduronate (Menini and Diczfalusy, 1960), estriol-3-sulfate (Menini and Diczfalusy, 1961) and 3β-hydroxyandrost-5-en-17-one (androstenolone) (Francis *et al.*, 1960) from this intestinal material. This report is concerned with the isolation, crystallization, and characterization of 3α-hydroxy-5β-pregn - 16 - en - 20 - one (16 - pregnenolone) from meconium.

EXPERIMENTAL

Source and Preliminary Fractionation of the Meconium.—16-Pregnenolone was isolated from pools of meconium collected from full-term male and female infants during the first 24 hours after birth. The procedures for the collection of the meconium and for the extraction, concentration, and hydrolysis of the conjugated steroids were described previously (Kinsella *et al.*, 1956). Ten kilograms divided into aliquots of 2,500 to 3,000 g of pooled meconium, which had been kept frozen during and after collection, were taken for analysis. Hydrolysis with bacterial β-glucosiduronidase was carried out as previously described. After incubation, the mixture was extracted with diethyl ether–chloroform (4:1) and with diethyl ether. These extracts were combined and evaporated to dryness. The residue was termed the "glucosiduronate fraction."

Separation into ketonic and non-ketonic fractions with Girard's T Reagent (Pincus and Pearlman, 1941) was carried out either on the "glucosiduronate fraction" directly or on the effluent from preliminary paper chromatography. In the latter instance, the "glucosiduronate fraction" was subjected to paper chromatography in the toluene–propylene glycol system (Burton *et al.*, 1951) for 12 days. The effluent solvent was evaporated and the residue was partitioned between carbon tetrachloride–diethyl ether (19:1) and 1 N KOH (Friedgood *et al.*, 1948). The residue of the carbon tetrachloride–diethyl ether extract was then treated with Girard's T Reagent. The ketonic material was further separated into α- and β-hydroxy fractions by the use of digitonin (Butler and Marrian, 1938).

Isolation of 16-Pregnenolone.—16-Pregnenolone was isolated from the α-hydroxy ketonic portion of the "glucosiduronate fraction" obtained from meconium. This fraction was chromatographed on Whatman No. 1 filter paper, 18 cm wide, in the petroleum ether (b.p. 64–67°)–propylene glycol system (Savard, 1953) for 18 to 24 hours at room temperature. Steroids used for reference, 17β-hydroxyandrost-4-en-3-one (testosterone), 3α-hydroxy - 5β - androstan - 17 - one (etiocholanolone), 3α - hydroxy - 5α - androstan - 17 - one (androsterone), and 3α - hydroxy - 5β - pregnan-20-one (pregnanolone), were applied as mixtures

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to 1-cm areas on the origin near both edges of the paper 2 cm distant from the centered area containing the residue. After the period of development, strips containing the reference compounds were cut lengthwise from the chromatogram, and a strip 2 mm wide was cut lengthwise from the center of the area of the paper to which the extract had been applied. Upon treatment of these strips with the Zimmermann reagents (Rosenkrantz, 1953), a major spot, orange in color, was noted in the area between androsterone and pregnanolone. This region of the chromatogram of the extract of meconium was eluted with absolute methanol and aliquots of the residue mixed with androsterone and mixed with pregnanolone were subjected to chromatography. After treatment of the strips with the Zimmermann reagents, the orange area was observed to have separated from the reference steroids. It had a rate of movement faster than that of androsterone and slower than that of pregnanolone.

In a few instances in which crystallization was not readily achieved directly from the residue of the eluate of the paper chromatogram, this residue was subjected to further purification. A partition between chloroform and water was performed in order to remove any traces of propylene glycol or paper which might be present. The residue of the chloroform extract was then chromatographed on a column of alumina. For example, a residue of 3.8 mg was transferred to a column 8 mm in diameter, containing 7.5 g of alumina,[1] with a total of 0.8 ml of benzene. Volumes of 50 ml of the following solvents were added successively: *n*-heptane, petroleum ether (b.p. 64–67°), 50% petroleum ether–benzene, benzene, 0.5% absolute ethanol–benzene, 1% absolute ethanol–benzene, and 2% absolute ethanol-benzene. Fractions of 25 ml were collected. The 1% absolute ethanol–benzene fractions contained a substance which, in 95% ethanol, exhibited an absorption maximum at 240 mμ. These fractions had been found, in control experiments, to contain 90% of authentic 16-pregnenolone which had been placed on the column.

Identification of 16-Pregnenolone.—The material eluted from the area of the chromatogram located between androsterone and pregnanolone or the residue of the material eluted in 1% absolute ethanol–benzene from the column of alumina contained a steroid which was identified as 3α-hydroxy-5β-pregn-16-en-20-one. After repeated crystallization from methanol - water, ethyl acetate–*n*-heptane, and ethyl acetate–petroleum ether, a product was obtained which melted at 202–204°.[2] An acetate was prepared which melted at 107–109°. The melting point of a mixture of the isolated material and authentic 3α-hydroxy-5β-pregn-16-en-20-one (m.p. 201–202°) was 203–205°. The melting point of a mixture of the acetate of the isolated material and authentic 3α-acetoxy-5β-pregn-16-en-20-one (m.p. 109.5–110.5°) was 107–109°.

The infrared spectra of the compound isolated from meconium and of its acetate were identical with those obtained for the corresponding authentic samples.[3] The absorption spectra in concentrated sulfuric acid were also identical. The isolated compound had an absorption peak at 240 mμ in 95% ethanol, gave no color in the Allen test (Allen *et al.*, 1950), and did not react to give color with blue tetrazolium.

Evaluation of the Effects of the Procedure of Girard T Separation.—An effort was made to evaluate the possible production of 16-pregnenolone in the course of the Girard separation of ketonic and non-ketonic substances. Two steroids with a hydroxyl group at carbon 16 or 17 and a ketonic group at carbon 20 were submitted in duplicate to the same Girard procedure as was used for the extract of meconium. To 2 mg of 3β,16α-dihydroxy-5β-pregnan-20-one or 3β,17α-dihydroxy-5α-pregnan-20-one were added 500 mg of recrystallized Girard's T Reagent and 2 ml of glacial acetic acid. The mixture was heated for 20 minutes, neutralized to *p*H 6.8 with iced 2.5 N NaOH, and extracted with diethyl ether. The aqueous phase containing the ketonic material was adjusted to *p*H 1 with concentrated HCl, allowed to stand for 2 hours at room temperature, and extracted with diethyl ether. The aqueous phase was then acidified to 1 N with concentrated HCl, allowed to stand overnight at room temperature, and re-extracted with diethyl ether. A reagent blank was carried through the entire procedure.

The Girard complex of 3β,16α-dihydroxy-5β-pregnan-20-one was hydrolyzed at *p*H 1 and with 1 N HCl. Neither fraction exhibited an absorption maximum at 240 mμ in 95% ethanol. This indicated that no dehydration to a Δ^{16}-20-ketone had occurred. When the absorption at 240 mμ of the ketonic material obtained after the *p*H 1 treatment of 3β,17α-dihydroxy-5α-pregnan-20-one was measured, there was an increase in absorption amounting to yields of 2.7 and 3% of a new conjugated system. The ketonic fraction obtained after acidification to 1 N HCl had no absorption peak at 240 mμ in 95% ethanol.

The α-hydroxy ketonic portion of the "glucosiduronate fraction" of meconium contained an appreciable amount of polar material which remained at the origin of the chromatogram after paper chromatography. It was conceivable that this material might yield 16-pregnenolone through the Girard treatment. Approximately 0.5 mg of this polar ketonic material, which did not have an absorption maximum at 240 mμ in 95%

[1] Alumina, Activated, Chromatographic, Catalyst Grade, Powdered, Harshaw Chemical Co., Cleveland, Ohio, was used as obtained.

[2] All melting point determinations were carried out with the Fisher-Johns apparatus.

[3] The infrared spectra were determined on a Perkin-Elmer Model 21 double-beam Spectrophotometer, with a KBr disc.

ethanol, was subjected again to the Girard procedure. No net gain of absorption at 240 mμ was obtained in the ketonic fraction after this second treatment with Girard's T Reagent, indicating that an α,β-unsaturated ketone had not been formed in appreciable amounts by the procedure.

Discussion

Although Fukushima *et al.* (1954) have previously detected and identified by infrared spectroscopy 3α-hydroxy-5β-pregn-16-en-20-one in the urine of a patient with Cushing's syndrome, this report is concerned with the first instance of the isolation and crystallization of this compound from meconium. Fukushima *et al.* (1954) and Neher *et al.* (1959) speculated as to whether the compound was excreted as such or was an artifact produced during hydrolysis or separation. It is not likely that this steroid was produced from a precursor by the mild methods used for hydrolysis, isolation, and purification. The results obtained with synthetic steroids and with extracts of meconium indicate that the fairly rigorous procedure of Girard separation was not responsible for the presence of 16-pregnenolone in extracts of meconium. In addition, we were unable to detect 16-pregnenolone in urine excreted by a patient with adrenal carcinoma although the aliquot of urine analyzed contained 65 mg of 17-hydroxycorticosteroids and 350 mg of 17-ketosteroids.[4] However, the method of Fukushima and co-workers (1954), which included initial treatment of urine with glucosiduronidase, should have been comparable to the method of hydrolysis employed in this study. Since the urinary studies may not be comparable, the likelihood remains that these investigators did isolate a metabolite rather than an artifact. Other factors, such as storage and effect of culture fluid, remain to be evaluated. The isolation of this steroid from meconium, a human source differing from that employed by Fukushima *et al.* (1954), would seem to offer evidence that it may be a genuine metabolite.

If the quantity of isolated compound is in itself significant, it would be of interest to compare the amount of 16-pregnenolone isolated from meconium with the amounts of other steroids isolated in earlier studies from this source. 16-Pregnenolone is present in meconium in a concentration of at least 1.3 mg per kg, estimated from the amount isolated. One kg of meconium contains about 0.5 mg of androstenolone, similarly estimated, the principal 17-ketosteroid, and 60 mg of estriol, also expressed as mg of isolated material, the principal estrogen. Since 16-pregnenolone is present in about twice the concentration of androstenolone, it assumes some quantitative significance.

The role of 16-pregnenolone in fetal-placental-maternal metabolism has yet to be assessed. The source of 16-pregnenolone is unknown, but experiments conducted in this laboratory[4] have shown that it is not a metabolite of 4,16-pregnadiene-3,20-dione after administration of this steroid to a normal adult man. Fukushima *et al.* (1954) have pointed out the likely relationship of 16-pregnenolone to 3α-16?-dihydroxy-5β-pregnan-20-one. Unsaturation in the 16,17 position provides an intermediate which might be expected to undergo cleavage of the side-chain at carbon 17 through a pathway similar to that followed by unsaturated fatty acids. Correlatively, the occurrence of 17-hydroxylation by a route other than by means of the action of 17-hydroxylase (Plager and Samuels, 1953) and the formation of C-19 and C-18 steroids with oxygen functions at carbon 16 and carbon 17 are implied. Burstein and Dorfman (1962), who demonstrated the formation of 3α-hydroxy-5α-androst-16-ene from 4-C^{14}-cholesterol and 7α-H^3-3β-hydroxypregn-5-en-20-one (7α-H^3-pregnenolone), have commented that the formation of a 16,17 unsaturated intermediate may not be ruled out. The isolation of 16-pregnenolone from meconium, together with its earlier detection in urine by Fukushima *et al.* (1954), lends new support to the importance of consideration of metabolic pathways in which this compound may be an intermediate.

[4] Unpublished observations.

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