patients suffering from various forms of adrenal dysfunction are presented.

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The Isolation of Oestrone from the Urine of Cows in Late Pregnancy

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The identification of the oestrogens secreted into the circulation of the cow, and also of those excreted, is of importance because it might lead to a greater understanding of the function of oestrogens in this species and in mammals generally. Since also it is known that different oestrogens are excreted by the human, the horse and the goat (Marrian, 1930; Marrian & Bauld, 1955; Girard, 1933; Klyne & Wright, 1956; Pincus & Thimann, 1955; Rodd, 1953), the interest in studying those of the cow is enhanced.

Pearlman, Rakoff, Cantarow & Paschkis (1947) have isolated oestrone, mainly present as the free phenol, from the gall-bladder bile of pregnant cows in amount approximating to 0.6 mg./l. Szego & Roberts (1946) and Pope & Roy (1953) have also reported oestrogenic activity in extracts of bovine pregnancy blood and colostrum respectively, in amounts equivalent to about 5 µg. of oestradiol-

17β/l. in each. Cow's urine, particularly during late pregnancy, also yields oestrogenic extracts (Smith, Dickson & Erb, 1956). We have confirmed these findings in initial experiments with blood, colostrum and urine, and have found urine of late pregnancy to be much the richest source of oestrogen; this has therefore been investigated first.

METHODS AND RESULTS

Materials and bioassay

Materials. Diethyl ether was shaken with aqueous FeSO₄-H₂SO₄ solution to decompose peroxides (Vogel, 1948), although in the final stages of the work peroxide-free ether (May and Baker Ltd.) was used without any treatment. Toluene was a 'low-in-sulphur' grade (Hopkin and Williams Ltd.). Inorganic reagents were of analytical reagent quality. Celite 545 (Johns-Manville and Co. Ltd.) was used for chromatographic columns and was recovered for re-use by heating in air at 600°. Alumina was washed

with water, then with methanol and heated at 100° for 19 hr. and 160° for 10 min.; the pH of a specimen shaken with water was then 7. Melting points were determined on a Kofler block.

Late-pregnancy urine was from Shorthorn, Friesian and Guernsey cows of the National Institute for Research in Dairying herd, all being more than 9 months' pregnant. Urine was obtained in batches from single urinations or, when more convenient, as 24 hr. collections from individual cows. Batches were then preserved with toluene and were processed individually. They were not stored for longer than 3 days.

Bioassay. Oestrogenic activity was determined by the mouse uterine-weight method of Pope & Roy (1953), the extracts being dissolved in 50% aqueous propylene glycol.

Chromatographic evidence for the presence of oestrone and more hydrophilic oestrogens in late-pregnancy urine

Isolation of the total phenol fraction. Urine (500 ml.) was mixed with toluene (1/10 vol.), brought to boiling point, then conc. HCl (1/7 vol.) was added and the mixture refluxed for 1 hr. It was then cooled to room temperature, saturated with NaCl and extracted with ether (4 × 1/4 vol.). The ethereal solution was washed with saturated aqueous NaHCO₃ (2 × 1/15 vol.), then with water (2 × 1/30 vol.) and dried over Na₂SO₄ and evaporated to dryness, yielding an extract (1.4 g.). This extract was dissolved in toluene (100 ml./g. of extract) and the solution extracted with aqueous N-NaOH (4 × 1/4 vol.). The alkaline extracts were pooled, brought to pH 8.0 with conc. HCl, saturated with NaCl and extracted with ether (4 × 1/3 vol.). The ethereal solution was dried over Na₂SO₄ and evaporated to dryness, yielding the total phenol fraction.

Removal of steam-volatile phenols. The total phenol fraction was mixed with water (100 ml.) and the water distilled off at approximately 15 mm. mercury pressure, leaving a residue of non-volatile phenols (I) with an oestrogenic activity greater than 0.04 mg. of oestradiol-17β equivalent/l. of urine.

Fractionation of non-volatile phenols. A partition-chromatographic column of 2.5 cm. diameter was made by the method of Martin (1949) from Celite 545 (80 g.) and bottom phase (40 g.) of the B(3) solvent system of Bush (1952). The solvent system had first been equilibrated at 28° and the column was packed and operated at this temperature. The remainder of the non-volatile phenols (I), four-fifths of the total, in solution in the minimum volume of top and bottom phases of the B(3) system, was then placed on this column and the top phase eluted. Fraction 1 of eluate was 150 ml., fractions 2–13 were each 50 ml., fractions 14–16 each 200 ml. Finally the column was 'stripped' free of organic matter by running methanol through it. The methanol strippings comprised fraction 17. Each fraction was evaporated to dryness and the residues were tested for oestrogenic activity. Activity was found in fractions 4–6 and in fractions 16 and 17. Fractions 4–6 contained a total of about 0.13 mg. of oestrogen/l. of urine, expressed as oestradiol-17β, and were further examined as described below. Examination of the more hydrophilic oestrogens of fractions 16 and 17 was postponed.

Separation of ketones by the Girard method. Fractions 4–6 were then combined and ketones separated from non-ketones by the method of Girard & Sandulesco (1936).

Acetic acid (0.5 ml.) was added to the combined fractions, followed by Girard T reagent (0.126 g.); and the mixture then heated at 100° for 30 min., cooled and added to water at 0° containing anhydrous Na₂CO₃ (0.41 g.) in solution. The resulting precipitate was extracted into ether (3 × 30 ml.); the ether phase washed with water (10 ml. at 0°) and dried over Na₂SO₄; then the ether was distilled off, yielding the non-ketone fraction (II). The aqueous phase, together with the water washing (10 ml.), was then mixed with conc. HCl (3 ml.) and left at 20° for 24 hr. It was then extracted with ether (3 × 20 ml.) and the ether solution washed with aqueous saturated NaHCO₃ (10 ml.) and dried over Na₂SO₄. Distillation of the solvent yielded the ketone fraction (III).

Paper chromatography. Aliquots (1/50) of (II) and (III) and 10 μg. of oestrone were run on Whatman no. 2 paper at 28° by the descending method with the B(3) solvent system of Bush (1952). After drying the paper, the solvent front (visible in ultraviolet light) was marked and the paper sprayed with aqueous 5% K₂CO₃ and dried at 40–50°. The paper was then sprayed with a diazonium solution prepared by the following method (Boscott, 1951). A 1% solution (1 ml.), cooled to 5°, of *p*-aminophenyl 2-diethylaminoethyl sulphone in aqueous 3.5 N-HCl was mixed with aqueous 5% NaNO₂ (5 ml.) also at 5°. The mixture was kept at 0° for 10 min., then aqueous 5% K₂CO₃ (20 ml. cooled to 0°) added, after which it was used immediately. The paper was then dried at 40–50° in air, when oestrone gave a pink spot (*R_F* 0.59). The ketonic fraction (III) gave a spot (*R_F* 0.58) identical in colour with that given by oestrone. Fraction (II) gave no such spot. The paper was then sprayed with aqueous N-KOH and dried at 40–50° in air, when the pink spots from oestrone and (III) both deepened to the same mauve colour. In ultraviolet light both spots appeared dark.

Isolation of oestrone from late-pregnancy urine

Urine (54 l.) was then processed in batches of up to 13 l. as collected, by methods similar to those described above. The paper-chromatogram method for detecting the oestrone-like substance was used frequently throughout the work. A sequence of these methods found to be satisfactory is described below.

The total phenol fraction (13.6 g.) from two batches of urine (21 l. in all) was shaken and warmed to 60° with water (500 ml.), toluene (100 ml.) and benzene (400 ml.). The solution was cooled to 20°, decanted from insoluble matter and the lower layer extracted with fresh benzene (500 ml.). The combined benzene-toluene layers, on evaporation of the solvent, yielded a red oil (5 g.), giving by the Girard method 1.7 g. of ketonic material which was then chromatographed on a partition column (5 cm. diameter) made from Celite 545 (400 g.). The paper-chromatogram technique was used to select the particular phenolic ketone fraction (IV) rich in the oestrone-like substance.

Further fractionation of product (IV) and similar material was then achieved either by vacuum sublimation or by chromatography on an alumina column. The latter method was more effective and is described in greater detail.

High-vacuum sublimation. In two experiments, fractions such as (IV) were sublimed in a simple molecular still at 10⁻⁶ mm. mercury pressure, 100° heating-bath temperature and with an approx. 0.5 cm. gap between material and condenser. The sublimate yielded crystals (V, 3.8 mg.) m.p. 247.5–249°. The *R_F* of this substance on paper chromatograms was equal to that of oestrone.

Chromatography on alumina. Products (IV) and (V) and similar material, totalling approximately 150 mg., were combined and extracted from benzene solution (100 ml.) into aqueous *N*-NaOH (4 × 25 ml.); the combined aqueous solutions were brought to pH 8.4 with conc. HCl and extracted with ether (4 × 50 ml.). The ether solution was washed with saturated aqueous NaHCO₃ (5 ml.), dried over Na₂SO₄ and the solvent evaporated, yielding a phenolic fraction (90 mg.) which was submitted to the Girard procedure, giving a partly crystalline phenolic ketone fraction (37 mg.). This material was chromatographed on an alumina column (5 g.; 0.5 cm. diameter). It was put on the column in benzene (15 ml.) and elution continued with benzene. Fractions 1 and 2 (200 ml., 50 ml.) yielded gummy matter on evaporation of the solvent, but fractions 3–7 (175, 200, 150, 150 and 50 ml.) yielded crystals, which appeared by paper chromatography to contain oestrone.

Fractions 3–7 when collected together weighed 13 mg. This crystallized from 1 ml. of 5% methanol in benzene in needles (8 mg.). Further fractional crystallization from ethyl acetate yielded: (1) needles (4 mg.), m.p. 247–253°; (2) crystals (1 mg.), m.p. 243–248°. Product (1) had m.p. 247–251° [when mixed with oestrone, m.p. 251–253° (L. Light and Co. Ltd.: synthetic specimen)], and had the same oestrogenic activity as oestrone by the Allen-Doisy test with rats and by the mouse uterine-weight test.

Products (1) and (2) were then mixed, dissolved in aqueous *N*-NaOH (4 ml.) by shaking and filtered through Whatman no. 50 paper; the paper was washed with aqueous *N*-NaOH (1 ml.). Dimethyl sulphate (1.7 ml.) and 10*N*-NaOH (1.5 ml.) were then added to the filtrate in small portions alternately. The crystalline precipitate (4.1 mg.) which formed was collected by filtration, dissolved in benzene and the solution filtered. The residue from the filtrate after evaporation crystallized from ethanol in almost colourless, rhombohedral prisms (VI, 3.5 mg.), m.p. 168–169.5°, undepressed when mixed with a specimen of oestrone methyl ether, m.p. 168–169.5° (VII). The infrared spectra of (VI) and (VII) in KBr disks, when recorded on a Grubb-Parsons double-beam spectrophotometer, were identical.

DISCUSSION

The presence of oestrone, and also of other unidentified oestrogens more hydrophilic than oestrone, in acid-hydrolysed urine from cows in late pregnancy is thus shown. It is not known whether these oestrogens are present in the original urine as the free phenols or as derivatives. Oestrone is, for example, present as the sulphate in pregnant mare's urine (Schachter & Marrian, 1938).

The results of paper chromatography of the phenolic ketone fractions from late-pregnancy bovine urine may be used to estimate the quantity of oestrone present. Visual comparison of the azo-dye spots from one phenolic ketone fraction with those from known quantities of oestrone indicated 0.36 mg. of oestrone/l. of urine. Similarly, in the extraction of 21 l. of urine described in detail it was estimated that approximately 5 mg. of oestrone was present in the phenolic ketone fraction (IV), or 0.24 mg./l. of urine. It seems likely that with a photo-

electric scanner oestrone could be estimated with at least ± 20% accuracy in 10–50 ml. specimens of late pregnancy urine. Some success has already been achieved in estimating isoflavones in this way as the azo-dye spots (Pope & Wright, unpublished work).

It was noted in the course of the work that the Bush (1952) chromatographic systems, when used in conjunction with the diazonium reagent described in this paper, were excellent for the separation and detection of the steroid oestrogens oestriol, oestradiol-17β and oestrone in quantities of about 5 μg. In fact 1 μg. of these steroids could be detected on the chromatograms without difficulty. For oestriol the *C* system (Bush, 1952) is convenient, with Whatman no. 2 paper at 28° and the descending method, *R_F* being 0.22; whereas for oestradiol and oestrone the *B* (3), or even better, the *B* (1) system, is suitable. In the *B* (1) system by the descending method at 28° and Whatman no. 2 paper, oestradiol has *R_F* 0.33, oestrone 0.69. It was found necessary to equilibrate the papers in the tanks for at least 3 hr. before running.

Chromatograms run at 28° on no. 2 paper by the descending method with *C* or *B* (1) system ran quickly and evenly (approximately 30 cm. in 4 hr.) and gave round spots. The *B* (3) system, containing the more volatile benzene in place of toluene, tended to give slower-running and uneven chromatograms, owing probably to slight temperature variations in the room having a greater effect on this more volatile system.

Two points of chemical interest were noted in the course of the work. When oestrone acetate was chromatographed on alumina, treated as described in this paper, with light petroleum, benzene and ether as eluting solvents, complete hydrolysis to oestrone occurred. Many other hydrolyses and other reactions are known to occur when organic compounds are chromatographed on alumina (Lederer & Lederer, 1953). The second observation was that the infrared spectra of different specimens of oestrone, when examined in the crystalline state in potassium bromide disks, were not identical. We attributed this to polymorphism, which, it is known (Kofler & Hauschild, 1934), does occur with oestrone. The phenomenon has already been noted with other steroids existing in polymorphic forms (Dickson, Page & Rogers, 1955), and is presumably due to differences in hydrogen bonding. We hope to examine the spectra of the three polymorphs of oestrone in more detail.

SUMMARY

Oestrone has been isolated from acid-hydrolysed urine of cows in late pregnancy in amounts of approximately 0.3 mg./l. At least one other more hydrophilic oestrogen is also present.

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Sterol Metabolism

1. THE BIOSYNTHESIS OF [¹⁴C]ERGOSTEROL IN *CARPENTELES BREFELDIANUM* DODGE

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Ergosterol, uniformly labelled with radiocarbon (¹⁴C), was required for studies on the absorption and metabolism of sterols in the guinea pig, preliminary results on which have already been published (Glover, Leat & Morton, 1954). The preparation of [¹⁴C]ergosterol having a high specific activity has been described by Hanahan & Al-Wakil (1952). They grew the yeast, *Saccharomyces cerevisiae*, on a coenzyme A-deficient medium for 24 hr. to produce cells with a low ergosterol content, an effect observed previously by Klein (1951). The deficient cells were then returned to a coenzyme A-enriched medium for 1 hr. and later incubated with labelled acetate for 24 hr. This procedure enabled the organism readily to synthesize the normal amount of ergosterol from the added acetate without appreciable dilution with acetate derived from cell material. The final yield, however, in terms of the absolute amount of ergosterol (0.12–0.2 % dry wt.), is small, and consequently the percentage recovery of the radioactivity is low.

Ergosterol having a very high specific activity was not essential for our studies but an improved yield of the ¹⁴C recovered as [¹⁴C]ergosterol from the [*methy*l-¹⁴C]acetate precursor was sought. Previous studies in this laboratory (Glover, 1949) on the ergosterol content of moulds indicated that, under certain cultural conditions, some contained more

than 1 % of ergosterol (based on the dry weight of the mycelium), an observation in agreement with the work of Zook, Oakwood & Whitmore (1944). The mould, *Carpenteles brefeldianum* Dodge, obtained from the Centraalbureau voor Schimmelcultuur, Baarn, Holland, was selected for the biosynthesis of [¹⁴C]ergosterol because (a) this organism produces at least 1.5 % of its dry weight as ergosterol with acetate in the medium, whereas *Saccharomyces cerevisiae* synthesizes less than 0.35 % under conditions for the biosynthesis of [¹⁴C]ergosterol from labelled acetate (Kodicek, 1955; Hanahan & Al-Wakil, 1952); and (b) the ergosterol is easily extracted with fat solvents from the dried thallus, whereas that in yeasts is tightly bound in the cell structure and is obtained quantitatively only after digesting the cells in strong alkali (Shakir, 1948; Shaw & Jefferies, 1953).

EXPERIMENTAL

Media and materials

Raulin-Thom medium used previously by Oxford, Raistrick & Simonart (1935) for the culture of the above mould contained excessive carbon for biosynthetic work with ¹⁴C-labelled compounds and had to be modified. Their medium and the one finally adopted by us are listed in Table 1. The chemicals used were of 'Technical' grade.