The Isolation of a Sixth Kober Chromogen from the Urine of Pregnant Women and its Identification as 18-Hydroxyoestrone

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In the course of column partition-chromatographic analysis of ketonic-phenolic fractions obtained from ether extracts of enzymically hydrolysed pregnancy urine, Kober-chromogenic material slightly more 'polar' in its chromatographic behaviour than 16 α -hydroxyoestrone (17-oxo-oestra-1:3:5-triene-3:16 α -diol) was regularly detected. The two possibilities were at first considered that this material might have arisen by 'leakage' of the chromatographically similar 16-epioestriol (oestra-1:3:5-triene-3:16 β :17 β -triol) into the ketonic fractions or by 'tailing' of the 16 α -hydroxy-oestrone fraction on the columns. However, when a concentrate of this Kober-chromogenic material was subjected to a second Girard separation and the resulting ketonic fraction rechromatographed in the same system nearly 60% of the starting material was recovered. This finding disposed of these two possibilities and it became evident that a hitherto unknown ketonic Kober chromogen (KC-6) had been detected.

Subsequently it was found that 'KC-6' was in fact a mixture of two different Kober chromogens; and in the present paper the isolation and complete characterization of one of these (KC-6A) is described. Brief preliminary accounts of the main findings reported here have been published (Loke, Watson & Marrian, 1957; Loke, Marrian, Johnson, Meyer & Cameron, 1958).

EXPERIMENTAL AND RESULTS

Methods

Hydrolysis and extraction of urine. The procedure used for preparing ketonic-phenolic fractions from enzymically hydrolysed urine was essentially similar to that described by Marrian, Watson & Panattoni (1957), but two modifications of this procedure were introduced: (i) as the result of a suggestion made to us by Dr J. R. K. Preedy (The London Hospital) Bradosol (5% solution of β -phenoxy-ethyltrimethylammonium bromide; Ciba Laboratories Ltd.) was added to the hydrolysed urine (1 ml. of Bradosol/l. of urine) immediately before the initial extraction with ether. This almost completely eliminated the formation of troublesome emulsions. (ii) To ensure that the ketonic-phenolic fractions were not contaminated with non-ketonic material the Girard separation with trimethylammoniumhydrazide chloride was carried out twice in succession.

Column-partition chromatography. Large-scale partition chromatograms on Celite columns with the solvent system methanol-water-hexane-benzene (70:30:20:80, by vol.) were carried out in a constant-temperature room (25°) as described by Marrian *et al.* (1957). The ketonic-phenolic concentrate from about 200 l. of urine was applied to each column and the eluate collected in 40 ml. fractions. Fractions 30-45, containing KC-6, were combined and evaporated to dryness. Since such KC-6 concentrates were usually contaminated with the 'tailings' of the KC-5 fraction (Marrian *et al.* 1957) they were always rechromatographed after combining into batches corresponding to 400-450 l. of urine.

Paper chromatography. Two solvent systems of the Bush (1952) type were employed throughout this work: system 4, methanol-water-chloroform-benzene (66:33:36:64, by vol.); system 8, methanol-water-hexane-benzene (70:30:50:50, by vol.). Whatman no. 42 papers, previously extracted exhaustively in a Soxhlet apparatus with a mixture of methanol, chloroform and benzene, were used.

Amounts of urinary concentrates or of pure substances containing 5-50 μ g. of Kober chromogen (calculated as oestriol) were applied in methanolic solution to the papers, which were then equilibrated in the tanks overnight at 18° and developed for about 6 hr. After drying in air the papers were sprayed with diluted (1 in 5) Folin-Ciocalteu reagent and the spots developed by exposure to ammonia vapour in a sealed tank.

Under the conditions employed rather variable R_F values were obtained with pure oestrogens, and accordingly standard substances were always run on the same papers as the unknowns. For system 4 the standards used were 17-epioestriol (R_F about 0.50) and oestriol (R_F about 0.15), and for system 8 oestrone (R_F about 0.50) was the standard.

Miscellaneous. The Kober reaction was carried out by the method of Brown (1952), as modified by Bauld (1954), with the 'oestriol reagent' described by the latter.

Melting points are uncorrected for emergent stem.

Evidence for the presence of two different Kober chromogens in KC-6

Preliminary experiments on the purification of KC-6 were carried out on 26 mg. of a chromatographically prepared concentrate obtained as a brown viscous oil from 400 l. of late-pregnancy urine. Paper chromatography with system 4 showed an intense well-defined spot with R_F about 0.50, and, since only faint spots appeared elsewhere on the chromatogram, it was assumed at first that the material might be nearly homogeneous. However, after reduction of a portion of

the original concentrate with sodium borohydride in methanolic solution and treatment of the product with Girard's reagent T (trimethylammonium-hydrazide chloride; Girard & Sandulesco, 1936) in the usual way to remove any unreduced ketonic material paper chromatography in the same system showed the presence of two main spots of about equal intensities. One of these had the same R_f as that of the unreduced material, and the other had R_f about 0.15.

From the main bulk of the original KC-6 concentrate a small amount of crystalline material was obtained by rapid leaching with chilled acetone. The acetone-soluble solid on treatment with 0.1 ml. of chloroform at -20° yielded a somewhat larger amount of crystalline material. The two crystalline fractions were combined and purified by leaching again with chloroform at -20° and finally recrystallizing from methanol-benzene. The nearly white crystalline product (6 mg.) had m.p. $247-250^\circ$ (sealed evacuated capillary). In the Kober reaction it gave a colour, the optical density of which at $512.5\text{ m}\mu$ was only 19% of that of the colour given by the same weight of oestriol. On paper chromatography (system 4) a single spot was detected which had the same R_f (about 0.50) as that of the main component in the original KC-6 concentrate.

A portion of the crystalline material was reduced with sodium borohydride in methanolic solution. On paper chromatography (system 4) the reduction product showed an intense spot with the same R_f as that of the unreduced material and a second faint spot with R_f about 0.15. In the Kober reaction the intensity of the colour given by the reduction product was about 200% greater than that of the colour given by the unreduced substance.

The amorphous fraction recovered from the original KC-6 concentrate soluble in acetone and chloroform gave a positive Kober reaction and, like the original concentrate, showed a main spot with R_f about 0.50 on paper chromatography with system 4. However, in marked contrast with the behaviour of the crystalline fraction, reduction by sodium borohydride of the amorphous fraction yielded a product which gave a Kober reaction of virtually the same intensity as that given by the unreduced material. Furthermore, on paper chromatography (system 4), the reduction product showed an intense spot with R_f about 0.15 and a much less intense spot with R_f 0.50.

On the basis of these findings it was concluded that the original KC-6 concentrate must have contained two different Kober chromogens which could not be distinguished from one another by paper chromatography in the solvent system used but which could be partially separated by leaching with acetone and chloroform. It seemed evident

that one of these, which was provisionally designated as KC-6A, can be reduced with sodium borohydride to form a product which has the same R_f in the solvent system used as the parent compound, but which is a much stronger Kober chromogen than the latter: the other, provisionally designated as KC-6B, yields a reduction product which is considerably more 'polar' in its chromatographic behaviour than the parent compound, but which is not a stronger Kober chromogen than the latter.

The evidence obtained indicates that the purified crystalline fraction must have been a nearly pure specimen of KC-6A contaminated with no more than a trace of KC-6B; the amorphous fraction which was more soluble in acetone and chloroform must have contained predominantly KC-6B, but also an appreciable amount of KC-6A.

Properties of KC-6A: provisional identification as 18-hydroxyoestrone

General. A KC-6 concentrate weighing 84 mg. was prepared from 504 l. of late-pregnancy urine. By leaching this twice in succession at -20° with 0.2 ml. volumes of chloroform a nearly white solid was obtained. After crystallization from methanol-benzene the product (11.8 mg.) was combined with 4 mg. of the material remaining from the previous batch (see above). The combined material was leached once with 0.2 ml. of chloroform at -20° and recrystallized once from methanol-benzene and once from ethanol. The final product was obtained in a yield of 5.7 mg. and had m.p. $255-257^\circ$ (sealed evacuated capillary), $[\alpha]_D^{19} + 146^\circ$ (c, 0.369 in ethanol), and ϵ_{281}^{2200} in ethanol (Found: C, 75.4; H, 7.9. Calc. for $C_{18}H_{22}O_3$: C, 75.5; H, 7.7%).

The KC-6A recovered from the optical-rotation determination was combined with 2.3 mg. of material recovered from the mother liquors of the analytical sample and acetylated in the usual way. The product was recrystallized twice from ethanol, yielding a product which had m.p. $162-165^\circ$ (hot stage) (Found: C, 70.8; H, 7.0. Calc. for $C_{22}H_{26}O_5$: C, 71.3; H, 7.1%).

In the Kober reaction the preparation of KC-6A gave a colour the intensity of which ($E_{512.5\text{ m}\mu}$) was 21% of that of the colour given by the same weight of oestriol. The intensity of the Kober colour given by the sodium borohydride reduction product of the preparation was 133% greater than that of the colour given by the unreduced substance. The absorption spectra of the Kober colours of KC-6A and of its reduction product are shown in Fig. 1. It will be seen that whereas the spectrum of the colour given by the reduction product is normal with a single maximum at $512.5\text{ m}\mu$, that of the colour given by KC-6A itself shows a second maximum at $420\text{ m}\mu$ of about the same intensity as that at $512.5\text{ m}\mu$.

The absorption spectra of solutions of KC-6A in conc. H_2SO_4 and of its sodium borohydride-reduction product after standing for 2 hr. at 24° are shown in Fig. 2. In the 'blue tetrazolium' test as carried out by Mader & Buck (1952) KC-6A showed negligible reducing power.

Since KC-6A is ketonic and has the probable formula $\text{C}_{18}\text{H}_{22}\text{O}_3$ and since it gave a positive Kober reaction the working hypothesis was adopted that it might be a 17-hydroxy or 17-oxo derivative of oestra-1:3:5-trien-3-ol with an oxo or hydroxy substituent elsewhere in the molecule. As KC-6A showed negligible reducing power towards 'blue tetrazolium' C-16 could be excluded as the other substituted position.

Since KC-6A and its sodium borohydride-reduction product had the same R_f in the solvent system

used it seemed possible that in the latter compound the two alcoholic hydroxyl groups might be influencing one another by steric hindrance or by hydrogen bonding. Substitution at C-16 had been excluded and the possibility of substitution at C-18 was therefore considered.

Infrared spectrum. Valuable further preliminary information about the structure of KC-6A was obtained from an infrared spectrum (KCl disk) which was determined by Dr R. K. Callow, National Institute for Medical Research.

In the carbonyl-stretching region there was a prominent band with a somewhat flattened peak at 1725 cm^{-1} . Since Meyer (1955) has reported carbonyl absorption at this frequency with certain 17-oxosteroids in KBr disks it seemed possible that this band in the KC-6A spectrum might be due to a 17-oxo group.

The presence of a band at 1408 cm^{-1} in the KC-6A spectrum provided support for this view, since Jones & Cole (1952) and Jones, Cole & Nolin (1952) have reported that a band at this frequency in steroids is associated with a 17-oxo group adjacent to an unsubstituted methylene group at C-16.

Jones & Cole (1952), Jones *et al.* (1952) and Kraychy & Gallagher (1957) have ascribed a band at $1375\text{--}1377\text{ cm}^{-1}$ in steroid spectra to the C-13 methyl group. No band at or close to this frequency was seen in the KC-6A spectrum and, accordingly, the possibility that in KC-6A there might be an oxygen substituent at C-18 was further considered.

Action of alkali on KC-6A. At this stage of the investigation the evidence, although far from conclusive, suggested that KC-6A might be 18-hydroxyoestrone.

Fig. 3. Action of sodium hydroxide on methyl hederagone and ietrogenin (I, partial formula) (Barton & de Mayo, 1954); hypothetical action of sodium hydroxide on 18-hydroxyoestrone (II, partial formula) with the formation of 18-noroeestrone (III, partial formula).

Fig. 1. Absorption spectra of colours given in the Kober reaction by KC-6A (●) and the sodium borohydride-reduction product of KC-6A (○).

Fig. 2. Absorption spectra, after 2 hr. at 24° , of a solution in sulphuric acid of KC-6A (●) and the sodium borohydride-reduction product of KC-6A (○).

Barton & de Mayo (1954) have shown that the primary β -ketols methyl hederagonate and icterogenin (Fig. 3, I) lose formaldehyde on treatment with alkali at room temperature. It seemed probable therefore that if KC-6A were 18-hydroxy-oestrone (Fig. 3, II) it should, by analogy, lose formaldehyde with the formation of 18-noroestrone or its C-13 epimer (Fig. 3, III) on treatment with alkali.

A solution of 100 μ g. of KC-6A in 2 ml. of *N*-NaOH was set aside at room temperature in a stoppered distillation flask. A control flask without KC-6A was also set up. After 4 hr. 3 ml. of 10*N*-H₂SO₄ and 4 ml. of water were added to each flask and the contents distilled into graduated test tubes each containing 1 ml. of 4% Na₂SO₃ solution. When the volumes of liquid in each tube were 5 ml. the distillations were stopped and the volumes made up to 7 ml. with water. To 2 ml. portions of each distillate was added 3 ml. of 0.2% chromotropic acid in 30*N*-H₂SO₄ and the mixtures were heated at 100° for 30 min. After cooling the colour developed in the 'experimental' tube was measured at 570 $m\mu$ against the 'control', and the amount of formaldehyde corresponding to the colour was read from a previously constructed calibration curve. The yield of the formaldehyde-like substance (9.45 μ g.) was 90% of that expected.

The solution remaining in the distillation flask was distributed between water and ether. The ether phase, after washing with 5% NaHCO₃ solution and water, was evaporated to dryness. One-fifth of the residue was chromatographed on paper with system 8, when a spot with the same *R_f* as that of oestrone was detected. A Kober reaction carried out on another portion of the residue was entirely negative.

Since on treatment with alkali KC-6A yielded a volatile substance resembling formaldehyde in nearly the expected amount and a product resembling oestrone in its chromatographic behaviour, KC-6A was provisionally identified as 18-hydroxy-oestrone and the oestrone-like product obtained from it as an 18-noroestrone (Loke *et al.* 1957).

Identification of the products formed from KC-6A by the action of alkali

Since the synthesis of 18-hydroxyoestrone did not seem to be immediately practicable it was decided to attempt to obtain proof of the structure of KC-6A by identification of the products formed from it by alkali treatment. At our request, Professor W. S. Johnson of the University of Wisconsin very kindly agreed to attempt the preparation of the two C-13 epimeric 18-noroestrone-3-methyl ethers for comparison with the methyl ether of the oestrone-like compound formed from KC-6A.

From 400 l. of late-pregnancy urine 16 mg. of crude KC-6A was prepared by similar methods to those described above. After three crystallizations from methanol well-formed crystals were obtained which showed a double melting point (about 220° and 248–257°; sealed evacuated capillary).

Material recovered from the mother liquors (11.2 mg.) was dissolved in 200 ml. of *N*-NaOH and the solution stood at room temperature. After 5 hr. the reaction mixture was acidified with HCl and extracted with chloroform. The extract was washed with 5% Na₂CO₃ solution and water, and dried over anhydrous Na₂SO₄ and evaporated to dryness. The product weighed 10 mg.

Identification of formaldehyde. The acidified aqueous phase from the experiment described above was distilled into 50 ml. of a cold saturated solution of 2:4-dinitrophenylhydrazine in 2*N*-HCl until the total volume was about 150 ml. The mixture was extracted with ether, and the extract washed thoroughly with 10*N*-H₂SO₄ and water, and dried over anhydrous Na₂SO₄ and evaporated to dryness. The residue was purified by filtering in benzene solution through a column of alumina, and then by crystallizing three times from ethanol. The product was obtained in fine yellow needles, m.p. 160–162.5° (hot stage). After admixture with authentic formaldehyde 2:4-dinitrophenylhydrazone (m.p. 162–164°) the m.p. was 161–163°.

Properties and identification of the oestrone-like product

The chloroform-extractable solid obtained from the alkali-treated KC-6A was leached once with 0.2 ml. of cold methanol to remove pigmented contaminants and crystallized from methanol, when 1 mg. of slightly yellow well-formed crystals was obtained, which partially melted with slight decomposition at 263°, resolidified at 265° and finally melted at 266–267° (sealed evacuated capillary). The product gave a negative Kober reaction. The absorption spectrum of a solution in conc. H₂SO₄ after standing for 2 hr. at 24° is shown in Fig. 4. It is noteworthy that this spectrum is virtually superimposable on that given by KC-6A under the same conditions.

The material recovered from the methanol mother liquor (9 mg.) was combined with 2 mg. of a similar product obtained from a previous batch of KC-6A and was methylated with dimethyl sulphate and *N*-NaOH at 37°. The mixture was extracted with ether and the extract washed with water and evaporated to dryness. The residue failed to give a pure product on crystallization from ethanol. Accordingly it was chromatographed in 33% (v/v) benzene in hexane on 1 g. of alumina. The main fraction from the chromatogram (4.9 mg.) was crystallized twice from methanol, when 1.3 mg.

Fig. 4. Absorption spectrum, after 2 hr. at 24°, of a solution in sulphuric acid of the 18-noroestrone obtained from KC-6A by treatment with sodium hydroxide.

of the methyl ether, m.p. 143–147° (hot stage), was obtained.

In the meantime, Professor Johnson and his co-workers had synthesized the two C-13 epimeric DL-18-noroestrone methyl ethers. The mixed m.p. of one of these (m.p. 145–147°, hot stage) with the methyl ether of the product obtained from KC-6A was 142–147°. The infra-red spectra of the two compounds in chloroform solution (kindly determined by Dr W. L. Meyer of the University of Wisconsin) were identical. Accordingly, the oestrone-like compound obtained from KC-6A by the action of alkali is an 18-noroestrone, and it follows that KC-6A was indeed correctly formulated as 18-hydroxyoestrone (Loke *et al.* 1958).

DISCUSSION

Before the present work the only steroids with oxygen substituents at C-18 obtained from animal sources have been aldosterone (11 β :21-dihydroxy-3:20-dioxopregn-4-en-18-al) isolated from adrenal glands by Simpson *et al.* (1954), 21-hydroxy-3:20-dioxopregn-4-ene-18-carboxylic acid (as the 18 $\rightarrow$ 20-hydroxylactone) isolated from adrenal glands by Neher & Wettstein (1956), and 3:20-dioxopregn-4-ene-18:21-diol obtained by Kahnt, Neher & Wettstein (1955) by incubating 11-deoxycortico-sterone (3:20-dioxopregn-4-en-21-ol) with adrenal homogenates. Since these compounds are all of adrenal origin it seems reasonable to suppose that 18-hydroxyoestrone may be formed in the adrenal glands by 18-hydroxylation of oestrone.

With this possibility in mind Loke *et al.* (1957) in preliminary experiments incubated oestrone with ox-adrenal homogenates and obtained small yields of a Kober chromogen which resembled 18-hydroxyoestrone in its chromatographic behaviour. On treatment with alkali the product yielded formaldehyde and a substance which resembled oestrone in its chromatographic behaviour. These preliminary findings support the view that 18-hydroxyoestrone may be formed in the adrenal glands.

The fact that sulphuric acid solutions of 18-hydroxyoestrone and of the 18-noroestrone formed from the latter have virtually identical absorption spectra suggests that, under the influence of the strong acid, formaldehyde is eliminated from 18-hydroxyoestrone as it is on alkali treatment. It is reasonable to assume that the same reaction might also occur during the first stage of the Kober reaction, and in this way the fact that 18-hydroxyoestrone is only weakly chromogenic in this reaction could be explained.

SUMMARY

1. A hitherto undescribed ketonic Kober chromogen (KC-6A) has been isolated from the urine of pregnant women in yields of 2–4 mg./100 l.

2. On treatment at room temperature with sodium hydroxide KC-6A yielded formaldehyde and a solid product which was not chromogenic in the Kober reaction but which resembled oestrone in its chromatographic behaviour.

3. This oestrone-like product obtained from KC-6A has been identified (as its methyl ether) as an 18-noroestrone. KC-6A is therefore 18-hydroxyoestrone.

4. 18-Hydroxyoestrone is accompanied in the ketonic-phenolic fractions of pregnancy urine by another Kober chromogen (KC-6B) which has similar chromatographic properties.

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Comparative Detoxication

6. THE METABOLISM OF 6-AMINO-4-NITRO-*o*-CRESOL AND 4:6-DINITRO-*o*-CRESOL IN LOCUSTS*

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4:6-Dinitro-*o*-cresol (DNOC) has been used for many years as an insecticide, especially against locust swarms in Africa (Rainey & Sayer, 1953). No chemical data on its metabolism in locusts are available, but it seems possible from toxicological experiments that it is detoxicated in these insects. McCuaig & Sawyer (1957) have shown that repeated sublethal doses of DNOC are not fully cumulative in locusts and point out that this can seriously diminish the effectiveness of field-spraying operations where locusts are sprayed over a period of several days.

Detoxication of DNOC in locusts could be accomplished by a reduction to 6-amino-4-nitro-*o*-cresol, such as is found in rabbits (Smith, Smithies & Williams, 1953), since it is known that locusts can reduce the aromatic nitro group (Friedler & Smith, 1954). We have therefore studied the fate of 6-amino-4-nitro-*o*-cresol in *Locusta migratoria* and *Schistocerca gregaria* as a preliminary to searching for this compound and its metabolites in locusts poisoned with DNOC.

Detoxication of a phenol like DNOC might also occur by conjugation reactions leading to either a β -glucoside or an arylsulphate (cf. Smith 1955*a*, *b*). Locust-crop fluid, however, contains an active β -glucosidase and aryl sulphatase (Robinson, Smith & Williams, 1953), and β -glucosidase has also been found in the intestinal wall of mid- and hind-gut (Robinson, 1956). If these enzymes were to mix with the excretions from the Malpighian tubes, which discharge into the posterior end of the

mid-gut of the locust, an initial conjugation might be reversed. We have therefore examined the contents of the different parts of the gut for β -glucosidase and aryl sulphatase in an attempt to assess their possible effect on any *O*-conjugated metabolites before these are eliminated in the excreta.

MATERIALS AND METHODS

Reference compounds. DNOC and other phenolic compounds were samples used in earlier work (Smith *et al.* 1953) or prepared by the same methods.

Glucosides of phenols were prepared by the general method of Glazer & Wulvek (1924). Tetra-acetyl- α -glucosyl bromide (34 g.) in 200 ml. of acetone was mixed with 6-acetamido-4-nitro-*o*-cresol (24 g.) in 150 ml. of water containing 6.4 g. of NaOH. After 24 hr. the acetone was removed *in vacuo* at 40–50° and the acetylated glucoside precipitated by addition of water to the residue. The precipitate was crystallized from ethanol to give 5 g. of 6-acetamido-4-nitro-*o*-cresyl tetra-acetyl- β -D-glucoside as pale-yellow needles, m.p. 169°; $[\alpha]_D^{25} = -1.8^\circ$ (c, 1 in CHCl_3) (Found C, 51.1; H, 5.0; N, 5.5. $\text{C}_{25}\text{H}_{28}\text{O}_{13}\text{N}_2$ requires C, 51.1; H, 5.2; N, 5.2%).

Deacetylation of this compound (5 g.) in 90 ml. of methanol with methanolic 2N-barium methylate (1.5 ml.) at 0° for 18 hr. gave a crystalline precipitate (3.5 g.) which was recrystallized from 90% (v/v) ethanol to give 6-acetamido-4-nitro-*o*-cresyl β -D-glucoside monohydrate, m.p. 147–155°; $[\alpha]_D^{25} = -6.1^\circ$ (c, 0.4 in 50%, v/v ethanol) (Found C, 46.2; H, 6.1; N, 7.7. $\text{C}_{18}\text{H}_{20}\text{O}_9\text{N}_2 \cdot \text{H}_2\text{O}$ requires C, 46.2; H, 5.7; N, 7.2%). The water was not lost at 100°. The glucoside had λ_{max} . 250, 291; ϵ_{max} . 11 700, 6900 in 0.1N-NaOH and λ_{max} . 240, 289; ϵ_{max} . 12 500 and 7500 in 0.1N-HCl.

* Part 5: Smith (1958).