- Doudoroff, M., Barker, H. A. & Hassid, W. Z. (1947). *J. biol. Chem.* **168**, 725.
- Durell, J. & Fruton, J. S. (1954). *J. biol. Chem.* **207**, 487.
- Edelman, J. (1954). *Biochem. J.* **57**, 22.
- Edman, P. & Jorpes, E. (1941). *Acta physiol. scand.* **2**, 41.
- Fishman, W. H. & Green, S. (1956). *J. Amer. chem. Soc.* **78**, 880.
- Foster, A. B. (1953). *J. chem. Soc.* p. 982.
- French, D. (1954). *Advanc. Carbohydr. Chem.* **9**, 149.
- French, D. & Knapp, D. W. (1950). *J. biol. Chem.* **187**, 463.
- Giri, K. V., Nigam, V. N. & Srinivasan, K. S. (1954). *Nature, Lond.*, **173**, 953.
- Haworth, W. N. & Wylam, B. (1923). *J. chem. Soc.* p. 3120.
- Hehre, E. J. (1951). *Advanc. Enzymol.* **11**, 330.
- Helferich, B. & Gootz, R. (1931). *Ber. dtsch. chem. Ges.* **64**, 109.
- Helferich, B. & Leete, J. F. (1942). *Org. Synth.* **22**, 53.
- Helferich, B. & Schäfer, W. (1926). *Liebigs Ann.* **450**, 229.
- Hérissey, H., Fleury, P., Wickström, A., Courtois, J. & Le Dizet, P. (1954). *C.R. Acad. Sci., Paris*, **239**, 824.
- Jermyn, M. A. & Thomas, R. (1953). *Aust. J. biol. Sci.* **6**, 70.
- Koenigs, W. & Knorr, E. (1901). *Ber. dtsch. chem. Ges.* **34**, 965.
- Kooiman, P., Roelofsen, P. A. & Sweeris, S. (1954). *Enzymologia*, **16**, 237.
- Lindberg, B. & Macpherson, J. (1954). *Acta chem. scand.* **8**, 985.
- Lloyd, P. F., Pon, G. & Stacey, M. (1956). *Chem. & Ind.* p. 172.
- Manners, D. J. (1955). *Biochem. J.* **61**, xiii.
- Matsuda, K. & Aso, K. (1953). *J. Ferment. Technol. (Japan)*, **31**, 211.
- Miller, L. P. (1943). *Contr. Boyce Thompson Inst.* **13**, 185.
- Miwa, T. & Takano, K. (1950). *Symp. Enzyme Chem. (Japan)*, **4**, 76. [Cited in *Chem. Abstr.* (1952). **46**, 2097.]
- Miwa, T., Takano, K., Mafune, K. & Furutani, S. (1949). *Proc. imp. Acad. Japan*, **25**, 111.
- Parr, C. W. (1954). *Biochem. J.* **56**, xxvii.
- Pazur, J. H. & French, D. (1952). *J. biol. Chem.* **196**, 265.
- Peat, S., Whelan, W. J. & Hinson, K. A. (1952). *Nature, Lond.*, **170**, 1056.
- Peat, S., Whelan, W. J. & Rees, W. R. (1956). *J. chem. Soc.* p. 44.
- Rabaté, M. J. (1935). *Bull. Soc. Chim. biol., Paris*, **17**, 572.
- Siu, R. G. H. (1951). *Microbial Decomposition of Cellulose*, p. 227. New York: Reinhold Publ. Corporation.
- Springhorn, S. S. & Koshland, D. E. (1955). *Abstr. Papers 128th Meeting, Amer. chem. Soc.* p. 37C.
- Stone, B. A. (1954). *Preparation of Cellulolytic and Related Enzymes and their Mode of Action on Cellulose and other β -glucosides*. Ph.D. Thesis: University of London.
- Takano, K. & Miwa, T. (1950). *J. Biochem., Tokyo*, **37**, 435.
- Takano, K. & Miwa, T. (1953). *J. Biochem., Tokyo*, **40**, 471.
- Thompson, A., Anno, K., Wolfrom, M. L. & Inatome, M. (1954). *J. Amer. chem. Soc.* **76**, 1309.
- Trevelyan, W. E. & Harrison, J. S. (1952). *Biochem. J.* **50**, 298.
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
- Veibel, S. (1936). *Enzymologia*, **1**, 124.
- Veibel, S. (1950). *The Enzymes*, vol. I, part I. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.
- Vintilescu, I., Ionescu, C. N. & Kizyk, A. (1934). *Bul. Soc. Chim. Român.* **16**, 151.
- Vintilescu, I., Ionescu, C. N. & Kizyk, A. (1935a). *Bul. Soc. Chim. Român.* **17**, 137.
- Vintilescu, I., Ionescu, C. N. & Kizyk, A. (1935b). *Bul. Soc. Chim. Român.* **17**, 283.
- Vintilescu, I., Ionescu, C. N. & Solomon, M. (1935). *Bul. Soc. Chim. Român.* **17**, 267.
- Whistler, R. L. & Durso, D. F. (1950). *J. Amer. chem. Soc.* **72**, 677.
- Wilham, C. A., Alexander, B. H. & Jeanes, A. (1955). *Arch. Biochem. Biophys.* **59**, 61.
- Wolfrom, M. L. & Thompson, A. (1955). *J. Amer. chem. Soc.* **77**, 6403.
- Zill, L. P., Khym, J. X. & Cheniae, G. M. (1953). *J. Amer. chem. Soc.* **75**, 1339.

The Isolation of a Ketonic Dihydroxy Kober Chromogen from the Urine of Pregnant Women

By G. F. MARRIAN, ELIZABETH J. D. WATSON AND MARTA PANATTONI

Department of Biochemistry, University of Edinburgh

(Received 3 May 1956)

In 1943 Pincus & Pearlman briefly referred in a review article to evidence obtained by them 'for the presence of an oestrogen in human pregnancy urine which is ketonic and contains a hydroxyl group other than the usual phenolic one'. Experimental details of this work were not published, but subsequently Huffman & Grollman (1947) suggested that this ketonic oestrogen might be 16-oxo-oestradiol-17 β (oestra-1:3:5-triene-3:17 β -diol-16-one), which previously had been prepared from

oestrone by Huffman (1942) (cf. Huffman & Lott, 1948); it was also suggested by these authors that 16-oxo-oestradiol-17 β might be an intermediate in the metabolic conversion of oestrone (oestra-1:3:5-trien-3-ol-17-one) into oestriol (oestra-1:3:5-triene-3:16 α :17 β -triol). More recently Migeon (1953) has reported the detection in extracts of acid-hydrolysed human urine of a H₂SO₄-fluorogen which behaved like 16-oxo-oestradiol-17 β on counter-current distribution in two different solvent

systems; but since 16-oxoestradiol-17 β added to urine could not be subsequently recovered after subsection of the latter to the usual acid hydrolysis and extraction procedures which were employed, it was concluded that the unidentified H₂SO₄-fluorogen was probably not identical with the former.

The suggestion that 16-oxoestradiol-17 β might be an intermediate in the metabolism of the oestrogens was recently readvanced by Marrian & Bauld (1955) when they isolated 16-epioestrinol (oestra-1:3:5-triene-3:16 β :17 β -triol) from the urine of pregnant women. Although, at that time, the possibility that the isolated substance was an artifact had not been excluded (cf. Watson & Marrian, 1956), it was nevertheless tentatively suggested that 16-oxoestradiol-17 β might be the common metabolic precursor of oestrinol and 16-epioestrinol.

Following up this suggestion Watson & Marrian (1955)* examined the urine of pregnant women for the presence of this hypothetical ketonic precursor of the two epimeric triols; they were able to detect a Kober chromogen in ether-soluble ketonic-phenolic fractions, which was more 'polar' than oestrone, and which was indistinguishable from 16-oxoestradiol-17 β by chromatography in the system 70 % (v/v) methanol in water on Celite-25 % (v/v) ethylene dichloride in benzene, and by countercurrent distribution in the system 20 % (v/v) *n*-hexane in benzene-50 % (v/v) ethanol in water. In these experiments preliminary enzymic hydrolysis of the urine gave yields of the unknown Kober chromogen about 2-3 times higher than those obtained after conventional hot-acid hydrolysis; and since 16-oxoestradiol-17 β was found to be labile to hot-acid treatment in urine this finding indicated a further similarity between the urinary Kober chromogen and this compound.

Although it was believed by Watson & Marrian (1955) that the unknown ketonic Kober chromogen (KC-5) was 16-oxoestradiol-17 β , it was appreciated that the identity of the former could only be established with certainty after isolation. The work described in the present paper was accordingly undertaken.

RESULTS

Isolation of KC-5

Partition chromatographic analysis of ether-soluble ketonic-phenolic fractions obtained from batches of pooled late-pregnancy urine after hydrolysis with enzymic preparations from *Patella vulgata* showed these fractions to contain 3.4-4.4 %

* The results obtained by Watson & Marrian (1955) were not reported in full detail. It is regretted that owing to limitations of space it is not possible to give these results in detail in the present paper.

by weight of oestrone and 14-22 % by weight of KC-5 (calc. as oestrinol) (Table 1). The most effective method of those tried for the further concentration of KC-5 in such fractions was found to be chromatography on a large Celite column with the system 70 % (v/v) methanol in water-20 % (v/v) *n*-hexane in benzene. By this method, for instance, a concentrate containing 82 % of KC-5 was obtained from batch III. The isolation of crystalline material from such concentrates was achieved by washing with a small volume of chilled methanol and then crystallizing from methanol at -20°. The yield of recrystallized material was poor, but further quantities of crystalline material were obtained from the methanolic washing and mother liquor by evaporation to dryness and re-treating with small volumes of chilled methanol.

The recrystallized KC-5 melted at 236.5-238.5°, and the melting point was not significantly depressed after admixture with 16-oxoestradiol-17 β (m.p. 238-241°). It gave C and H analyses in excellent agreement with those required for a compound of the formula C₁₈H₂₂O₃. However, it was clear that KC-5 could not be identical with 16-oxoestradiol-17 β since it had $[\alpha]_D^{15} + 143^\circ$ in ethanol (cf. 16-oxoestradiol-17 β , $[\alpha]_D^{15} - 89^\circ$ in ethanol). Furthermore, in contrast to 16-oxoestradiol-17 β , it developed its maximum colour in the Kober reaction with no heating in the first stage (Fig. 1),

Table 1. Ketonic-phenolic fractions from enzyme-hydrolysed late-pregnancy urine: oestrone and KC-5 contents

Batch	Vol. of urine (l.)	Wt. of ketonic-phenolic fraction (mg.)	Oestrone content of fraction (%)	KC-5 content (%)
I	75	207	3.5	15
II	101	377	3.5	14
III	100	284	3.4	22
IV	95	274	4.4	15

Fig. 1. Effect of variation in time of heating in the first stage of the Kober reaction on intensities of final colours developed by 16-oxoestradiol-17 β (●—●) and KC-5 (●- -●).

Fig. 2. Absorption spectra of H_2SO_4 solutions of 16-oxoestradiol-17 β and KC-5 after 2 hr. at 24°. A, 16-Oxoestradiol-17 β : $8.39 \times 10^{-5}\text{M}$; B, KC-5: $8.74 \times 10^{-5}\text{M}$.

Fig. 3. Ultraviolet absorption spectra of 16-oxoestradiol-17 β and KC-5 in ethanolic solution. A, 16-Oxoestradiol-17 β : $1.67 \times 10^{-4}\text{M}$; B, KC-5, $1.75 \times 10^{-4}\text{M}$.

while the spectrum of its solution in conc. H_2SO_4 differed markedly from that of a similarly prepared solution of 16-oxoestradiol-17 β (Fig. 2).

Characterization of KC-5

General. On acetylation KC-5 yielded a diacetate which melted at 166–167° (cf. 16-oxoestradiol-17 β diacetate, m.p. 132.5–134°). This suggested the possibility that KC-5 might be 6-oxoestradiol-17 β , the acetate of which melts at 173–175° (Longwell & Wintersteiner, 1940). However, this possibility was excluded by the ultraviolet-absorption spectrum in ethanol of KC-5, which was found to be virtually identical with those of 16-oxoestradiol-17 β (Fig. 3), oestrinol, 16-epioestrinol and oestrone, but markedly

different from that of 6-oxoestradiol-17 β as recorded by the above-mentioned authors.

In the 'blue tetrazolium' test carried out according to Mader & Buck (1952), KC-5 showed marked reducing power, suggesting that it was an α -ketol. However, the intensity of the colour developed in this test was only about one-third of that of the colour developed by an equivalent amount of 16-oxoestradiol-17 β . This finding suggested the possibility that KC-5 might be a 16-hydroxyoestrone, since it had previously been found (Fotherby & Marrian, unpublished work) that 16 α -hydroxyandrost-4-ene-3:17-dione (Fried, Thoma, Perlman, Herz & Borman, 1955) gives a considerably less intense colour in this test than those given by either 16-oxoestradiol-17 β or pregn-4-ene-17 α :21-diol-3:20-dione. The dextro-rotation of KC-5 was consistent with the presence of a 17-oxo group (cf. Barton & Klyne, 1948), while the finding that the compound gave a negative Zimmermann reaction was consistent with the presence of 16-hydroxy-17-oxo grouping.

Sodium borohydride reduction of KC-5. To investigate the possibility of KC-5 being one or other of the two epimeric 16-hydroxyoestrones a sample of the isolated substance was reduced with sodium borohydride. Since by this method of reduction oestrone yields oestradiol-17 β exclusively (Biel, 1951), it was expected that KC-5 would yield either oestrinol or 16-epioestrinol, depending on whether the C-16 hydroxyl group of the former had the α - or β -configuration. Unfortunately, owing to the shortage

of available material this experiment had to be carried out on a sample of KC-5 which had been recovered from the mother liquors of the analytical sample.

On chromatographing a portion of the reduction product in the system 70% (v/v) methanol in water-ethylene dichloride the presence of two components in approximately equivalent amounts was unexpectedly revealed, and the elution characteristics of the less and more 'polar' of these were closely similar to those of 16-*epio*oestriol and oestriol respectively (Fig. 4). In view of this finding the main bulk of the reduction product was separated into its two components on a larger partition column with the same solvent system.

After recrystallization the less 'polar' component melted at 273–276°, and the melting point was not depressed after admixture with 16-*epio*oestriol (m.p. 284–285°). Because of the considerable difference in the melting points this was not accepted as completely satisfactory evidence of the identity of this reduction product with 16-*epio*oestriol. However, on acetylation the reduction product yielded an acetate, m.p. 151–152°, and the mixed melting point of this with 16-*epio*oestriol triacetate (m.p. 151–152°) showed no depression. Accordingly, there could be no doubt that this reduction product was identical with 16-*epio*oestriol.

The more 'polar' reduction product of KC-5 melted at 277–279° after recrystallization, and the melting point was not depressed after admixture with oestriol (m.p. 279–280°). It yielded an acetate, m.p. 125–128°, and the mixed melting point with oestriol triacetate (m.p. 126–128°) showed no depression. Accordingly, this second reduction product of KC-5 was clearly identical with oestriol.

On the reasonable assumption that no inversion of either a C-16 or a C-17 hydroxyl group occurs during sodium borohydride reduction, the only α -

ketol which could give rise to oestriol when reduced by this method is 16 α -hydroxyoestrone. Accordingly, it can be concluded that the latter was present in the sample of KC-5 used in the reduction experiment.

On the other hand, either 16 β -hydroxyoestrone or 16-oxooestradiol-17 β could give rise to 16-*epio*oestriol by sodium borohydride reduction, and, indeed, it has been shown by Huffman & Lott (1955) that 16-*epio*oestriol is formed in nearly quantitative yield when 16-oxooestradiol-17 β is reduced by this procedure. It seems probable, therefore, that the sample of KC-5 used in the reduction experiment was a mixture either of 16 α -hydroxyoestrone and 16 β -hydroxyoestrone or of 16 α -hydroxyoestrone and 16-oxooestradiol-17 β , although the alternative explanation, that a partial rearrangement of 16 α -hydroxyoestrone to 16-oxooestradiol-17 β occurred during the reduction, cannot be excluded. As the sample of KC-5 used for the reduction was material recovered from the mother liquors of the analytical sample, it is not unlikely that it was grossly impure. The diacetate of KC-5, which was prepared from a mixture of recrystallized material and material recovered from the mother liquors, had a melting point 13° lower than that reported for synthetic 16 α -hydroxyoestrone diacetate by Leeds, Fukushima & Gallagher (1954). This finding is consistent with the view that the KC-5 recovered from the mother liquors contained in addition to 16 α -hydroxyoestrone a considerable proportion either of 16 β -hydroxyoestrone or of 16-oxooestradiol-17 β .

While there is no reason to believe that the recrystallized KC-5 was a pure substance, it seems probable that the principal component in it must have been either 16 α -hydroxyoestrone or 16 β -hydroxyoestrone, and not the laevorotatory 16-oxooestradiol-17 β . If the principal component in it was 16 α -hydroxyoestrone, the accompanying impurity, which would presumably become concentrated in the mother liquor, could have been either 16 β -hydroxyoestrone or 16-oxooestradiol-17 β . On the other hand, if the principal component in it was 16 β -hydroxyoestrone, the accompanying impurity must have been 16 α -hydroxyoestrone.

Until a larger amount of more rigorously purified KC-5 has been examined a definite decision between these possibilities cannot be made. However, since Cooley, Ellis, Hartley & Petrov (1955) have shown that alkaline hydrolysis at room temperature of androst-5-ene-3 β :16 α -diol-17-one diacetate yields androst-5-ene-3 β :17 β -diol-16-one it is not improbable that if 16 α -hydroxyoestrone had been present in the original urinary extracts the analogous rearrangement of 16 α -hydroxyoestrone to 16-oxooestradiol-17 β might have occurred to some extent during the extraction of the phenolic fraction with aqueous alkali. The view that the isolated KC-5 was

Fig. 4. Analytical column partition chromatograms (70% methanol in water-ethylene dichloride) on A, sodium borohydride reduction product from KC-5, and B, a mixture of 15.5 μ g. of 16-*epio*oestriol and 16.3 μ g. of oestriol.

a mixture of 16 α -hydroxyoestrone and artifactually produced 16-oxooestradiol-17 β would seem to be entirely consistent with all the available evidence, and is therefore the favoured one.

EXPERIMENTAL

Methods

Enzymic hydrolysis of urine. Urine specimens were acidified to pH 4.7 with acetic acid, buffered to this pH by the addition of one-tenth of their volume of M acetate buffer, and incubated at 37° with the enzyme preparation for a period of 48 hr. In every instance half of the total amount of enzyme used was added initially and half after 24 hr.

Analytical partition chromatograms. The procedures based on those of Bauld (1955) and described by Marrian & Bauld (1955) and Watson & Marrian (1956) were employed.

Kober reaction. The method of Brown (1952), as modified by Bauld (1954), was employed, Bauld's 'oestriol reagent' being used. All determinations of Kober chromogens are expressed as oestriol equivalents.

Melting points. The melting points of 16-oxoestradiol-17 β , KC-5, oestriol and 16-epioestril were determined in sealed, evacuated capillary tubes, while for those of the acetates of these substances a microscope hot-stage was employed. The same thermometer was used for all determinations and the values given are uncorrected for emergent stem. Although corrected melting points were given for oestriol and 16-epioestril in a recent paper by two of the present authors (Watson & Marrian, 1956) we are now of the opinion that the method of correcting for emergent stem with the aid of pure standard compounds which was used is unreliable for temperatures much above 200°, because of the lack of reliable data for corrected melting points on suitable compounds.

Materials

16-Oxoestradiol-17 β . This was prepared from oestrone by the method of Huffman (1942) and Huffman & Lott (1948), and was crystallized once from 50% (v/v) aqueous acetic acid and once from dilute methanol. The m.p. was 239–241° and it had $[\alpha]_D^{25} - 89^\circ$ (0.600% in ethanol). It yielded a diacetate, m.p. 132.5–134°.

Oestriol. The preparation described by Marrian & Bauld (1955) was used. The m.p. was 279–280° and it had $[\alpha]_D^{19} + 61^\circ$ (0.296% in ethanol). It yielded a triacetate, m.p. 126–128°.

16-epioestril. The preparation described by Watson & Marrian (1956) was used. The m.p. was 284–285°, and it had $[\alpha]_D^{15} + 76^\circ$ (0.297% in ethanol). It yielded a triacetate, m.p. 151–153°.

Enzyme preparations. These were crude acetone-dried powders obtained from *Patella vulgata* by the method of Dodgson & Spencer (1953). They were standardized for β -glucuronidase activity with phenolphthalein glucuronide as substrate by the method of Fishman (1948). The sulphatase activities of these preparations were not determined.

Miscellaneous. The solvents and Celite (Celite 535, Johns Manville and Co., Ltd., London) used were purified by the same methods employed by Watson & Marrian (1956).

Isolation of KC-5 from pregnancy urine

Late-pregnancy urine was enzymically hydrolysed in batches of 8–16 l. with amounts of the enzyme equivalent to

about 1000000 Fishman units/l. of urine. The hydrolysed mixture was extracted once with an equal volume of ether after the addition of 600 g. of NaCl/4 l. of urine, the extract washed once with one-tenth of its volume of 5% (w/v) NaHCO₃ and then extracted twice with one-eighth volume of N-NaOH. The NaOH extract was partly neutralized by the addition of 5N-H₂SO₄, and then neutralization (to phenolphthalein) completed by passing in CO₂. The extraction with NaOH and the subsequent neutralization of the extract were carried out as rapidly as possible to minimize losses of KC-5 which occur in strongly alkaline solutions.

The neutralized extract was extracted twice with equal volumes of ether, the extract washed twice with one-quarter volumes of water, dried over Na₂SO₄ and evaporated to dryness.

Phenolic fractions obtained in this way were stored at 0° until 100 l. of urine had been processed. They were then combined and treated at room temperature for 17 hr. with 5 g. of trimethylammonium hydrazide chloride in 50 ml. of ethanol and 10 ml. of acetic acid. The ketonic-phenolic fraction obtained from the reaction mixture in the usual way weighed 284 mg., and by carrying out an analytical partition chromatogram on one-thousandth of the total amount it was shown to contain 9.7 mg. of oestrone and 63.4 mg. of KC-5.

The main bulk of the phenolic-ketonic fraction was chromatographed on a large column (2 cm. diam.) with 120 g. of Celite and the solvent system 70% (v/v) methanol in water–20% (v/v) *n*-hexane in benzene. Thirty successive 40 ml. fractions of the eluate were collected and evaporated to dryness, and Kober reactions carried out on small portions of each. Fractions 19–22, which contained the KC-5, were combined, yielding 55.3 mg. of a slightly pigmented solid which was found to contain 45.5 mg. of Kober chromogen. By washing twice with methanol chilled to –20° a white solid (35.0 mg.) was obtained. After this had been combined with 15.7 mg. of similarly prepared material from another batch of urine it was recrystallized from methanol at –20°, yielding 9.2 mg. of 'Grade I KC-5'. After drying for 2 hr. at 100° *in vacuo* the product had: m.p. 236.5–238.5°; $[\alpha]_D^{15} + 143^\circ$ (c. 0.254% in ethanol); ϵ_{230} , 2238 in ethanol (Fig. 3). (Found: C, 75.5; H, 7.9. Calc. for C₁₈H₂₂O₃: C, 75.5; H, 7.8%.)

By further treatment of the material obtained by evaporation of the methanol washings and mother liquors by rewashing with chilled methanol, 24.6 mg. of 'Grade II KC-5', m.p. 235–238°, and 15.3 mg. of 'Grade III KC-5', m.p. 234–238°, were obtained.

Properties and characterization of KC-5

Kober reaction on KC-5 and 16-oxoestradiol-17 β : variation in time of heating in first stage of reaction. The technique used in this experiment was the same as that described by Marrian & Bauld (1955) for a similar experiment with 16-epioestril (KC-4) and oestriol. For the Kober reactions on KC-5, 10 μ g. of the latter (Grade I) was used in each tube, and, for those on 16-oxoestradiol-17 β , 11 μ g. was used. The curves showing the relationship between optical density at 512.5 m μ . [corrected by using the Allen (1950) formula] are shown in Fig. 2.

Reaction with conc. H₂SO₄ on KC-5 and 16-oxoestradiol-17 β . To each of two tubes containing respectively 100 μ g. of KC-5 (grade I) and 96 μ g. of 16-oxoestradiol-17 β was added 4 ml. of conc. H₂SO₄, and the mixtures were allowed

to remain at 24° for 2 hr. with occasional shaking. Absorption spectra over the range 230–530 m μ . were determined on both solutions (Fig. 3). It is noteworthy that on adding the H₂SO₄ to the KC-5 an intense red colour developed immediately. This was not observed with 16-oxo-oestradiol-17 β .

KC-5 diacetate. KC-5 (6.7 mg. of grade I + 8.4 mg. of grade III) was treated at room temperature for 24 hr. with 0.5 ml. of acetic anhydride and 0.5 ml. of anhydrous pyridine. After the addition of ice and water, the crude acetate was filtered off, washed thoroughly with water, dried, and crystallized once from a mixture of ethyl acetate and *n*-hexane. The product melted at 166–167°, and the mixed m.p. with 16-oxo-oestradiol-17 β diacetate (m.p. 132.5–134°) was 120–142°. After drying for 1 hr. at 80° *in vacuo* the product had $[\alpha]_D^{25} +156^\circ$ (c. 0.301% in ethanol). (Found: C, 71.2; H, 6.8. Calc. for C₂₂H₂₀O₅: C, 71.3; H, 7.1%.)

Sodium borohydride reduction of KC-5. To a solution of 20 mg. of KC-5 (Grade II) in 10 ml. of methanol was added 15 mg. of sodium borohydride. After standing at room temperature for 40 min. a further 10 mg. of sodium borohydride was added and the mixture allowed to stand for a further 60 min. About half of the methanol was evaporated off under reduced pressure in a warm-water bath, and after dilution with 100 ml. of water and acidification with 4 ml. of 10*N*-HCl the mixture was extracted thoroughly with ether. The ethereal extract was washed with aqueous NaHCO₃ and with water, and evaporated to dryness. The product was treated at room temperature for 17 hr. with 250 mg. of trimethylammonium hydrazide chloride in 5 ml. of ethanol and 5 ml. of acetic acid, and the non-ketonic material was obtained in the usual way by ether extraction after dilution with water and partial neutralization with NaOH. The non-ketonic reduction product weighed 20.2 mg.

One five-hundredth of the product was chromatographed in the system 70% (v/v) methanol in water–ethylene dichloride, and the results indicated the presence in the total product of about 7.9 mg. of a less ‘polar’ Kober chromogen and about 9.6 mg. of a more ‘polar’ one. For comparison a mixture of 15.5 μ g. of 16-*epio*oestrinol and 16.3 μ g. of oestrinol were chromatographed in the same system (Fig. 4).

The main bulk of the non-ketonic reduction product was separated into its two components on a large partition column with 120 g. of Celite and the same solvent system. The less and more ‘polar’ fractions obtained weighed 6.4 and 7.8 mg. respectively.

After one crystallization from methanol–benzene the less ‘polar’ product melted at 273–276°, and the mixed m.p. with 16-*epio*oestrinol (m.p. 284–285°) was 274–281°. The remainder of the recrystallized material, together with that obtained by evaporation of the mother liquor, was acetylated in the usual way with acetic anhydride and pyridine at room temperature. The product, after one crystallization from *n*-hexane, melted at 151–152° and the mixed m.p. with 16-*epio*oestrinol triacetate (m.p. 151–153°) was 150–152°. The identification of the less ‘polar’ reduction product of KC-5 as 16-*epio*oestrinol was therefore considered to be satisfactory.

The more ‘polar’ reduction product melted at 277–279° after one crystallization from methanol–benzene, and the mixed m.p. with oestrinol (m.p. 279–280°) was 277–280°.

DISCUSSION

The average yield of KC-5 found in pooled late-pregnancy urine, as determined by analytical

partition chromatograms on the ketonic–phenolic fractions, was 0.5 mg./l.; and, since the extraction and fractionation processes employed were certainly not quantitative, it is probable that the average concentration of KC-5 actually present in urine is considerably higher. It is clear, therefore, that this newly discovered α -ketolic Kober chromogen is an oestrogen metabolite of considerable quantitative importance. Although it is present in late-pregnancy urine in such comparatively large amounts, it is hardly surprising, in view of its considerable lability to certain of the processes commonly employed in urine extraction and fractionation, that it has not hitherto been isolated. Rather it is surprising that it was detected in urine extracts by Watson & Marrian (1955), and also, presumably by Pincus & Pearlman (1943) and by Migeon (1953), in experiments in which conditions for its survival must have been far from optimum.

Although it is probable that the material isolated was not a pure substance, convincing evidence has been obtained which shows that the principal component in it must have been either 16 α -hydroxyoestrone or 16 β -hydroxyoestrone. While other evidence, which has already been discussed, is consistent with the view that the isolated KC-5 was composed of 16 α -hydroxyoestrone and some artifactually produced 16-oxo-oestradiol-17 β , the results of the present investigation indicate that 16-oxo-oestradiol-17 β is probably not present as such in urine. Accordingly the speculative suggestion advanced by Marrian & Bauld (1955) that 16-oxo-oestradiol-17 β might be the common metabolic precursor of oestrinol and 16-*epio*oestrinol receives no support.

The view that the principal component in KC-5 is 16 α -hydroxyoestrone rather than 16 β -hydroxyoestrone can be supported on biogenetic grounds. It now seems reasonable to suppose that both of these compounds may be formed metabolically from oestrone by 16 α - and 16 β -hydroxylation, and that they are intermediates in the ‘hydration’ of oestrone to oestrinol and 16-*epio*oestrinol respectively. Since the concentration of oestrinol in late-pregnancy urine is about 60 times that of 16-*epio*oestrinol, it might be assumed that the concentration of the immediate precursor of oestrinol would be much greater than that of the immediate precursor of 16-*epio*oestrinol.

SUMMARY

1. An α -ketolic Kober chromogen (KC-5) has been isolated from the urine of pregnant women.
2. The evidence obtained shows that the principal component in KC-5 was either 16 α -hydroxyoestrone or 16 β -hydroxyoestrone. Reasons for favouring the first alternative are given.

The authors wish to thank the Medical Research Council for a grant from which the expenses of this work were defrayed, E. R. Squibb and Sons for a generous gift of oestrone, Professor R. J. Kellar and Dr Susanne Paterson of the Department of Obstetrics and Gynaecology and Dr A. Klopper of the Clinical Endocrinology Research Unit (M.R.C.) for providing facilities for the collection of urine, and Dr J. W. Minnis for carrying out the microanalyses.

REFERENCES

- Allen, W. M. (1950). *J. clin. Endocrin.* **10**, 71.
 Barton, D. H. R. & Klyne, W. (1948). *Chem. & Ind.* p. 755.
 Bauld, W. S. (1954). *Biochem. J.* **56**, 426.
 Bauld, W. S. (1955). *Biochem. J.* **59**, 294.
 Biel, J. H. (1951). *J. Amer. chem. Soc.* **73**, 847.
 Brown, J. B. (1952). *J. Endocrin.* **8**, 196.
 Cooley, G., Ellis, B., Hartley, F. & Petrov, V. (1955). *J. chem. Soc.* p. 4373.
 Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **55**, 315.
 Fishman, W. H. (1948). *J. biol. Chem.* **173**, 449.
 Fried, J., Thoma, R. W., Perlman, D., Herz, J. E. & Borman, A. (1955). *Recent Progr. Hormone Res.* **11**, 149.
 Huffman, M. N. (1942). *J. Amer. chem. Soc.* **64**, 2235.
 Huffman, M. N. & Grollman, A. (1947). *Endocrinology*, **41**, 12.
 Huffman, M. N. & Lott, M. H. (1948). *J. biol. Chem.* **172**, 325.
 Huffman, M. N. & Lott, M. H. (1955). *J. biol. Chem.* **215**, 627.
 Leeds, N. S., Fukushima, D. K. & Gallagher, T. F. (1954). *J. Amer. chem. Soc.* **76**, 2943.
 Longwell, B. & Wintersteiner, O. (1940). *J. biol. Chem.* **133**, 219.
 Mader, W. J. & Buck, R. R. (1952). *Analyt. Chem.* **24**, 666.
 Marrian, G. F. & Bauld, W. S. (1955). *Biochem. J.* **59**, 136.
 Migeon, C. J. (1953). *J. clin. Endocrin. Metab.* **13**, 674.
 Pincus, G. & Pearlman, N. H. (1943). *Vitam. & Horm.* **1**, 334.
 Watson, E. J. D. & Marrian, G. F. (1955). *Biochem. J.* **61**, xxiv.
 Watson, E. J. D. & Marrian, G. F. (1956). *Biochem. J.* **63**, 64.

The Occurrence of *n*-Heptadecanoic Acid (Margaric Acid) in Unhydrogenated Mutton Fat

By R. P. HANSEN, F. B. SHORLAND AND N. JUNE COOKE

Fats Research Laboratory, Department of Scientific and Industrial Research, Wellington, New Zealand

(Received 5 June 1956)

When margaric acid was formerly isolated from mutton fat (Hansen, Shorland & Cooke, 1954) hydrogenation was employed in the method adopted. Accordingly it was not established whether this acid occurred as such in the natural fat, or whether it resulted (in part or in whole) from hydrogenation of either a C_{17} unsaturated acid or of a cyclopropane ring (cf. Hofmann & Lucas, 1950; Hofmann, Lucas & Sax, 1952). As Δ^9 -heptadecenoic acid has been identified in lamb-caul fat (Shorland & Jessop, 1955) its presence in mutton fat is expected, and this, on hydrogenation, could account for at least part of the margaric acid present. On the other hand, cyclopropane rings have not been shown to occur in natural glycerides, although, as reported by Hofmann *et al.* (1952), they are present in the lipids of certain species of *Lactobacillus*, and on hydrogenation they yield a methyl-branched-chain acid and a normal odd-numbered fatty acid.

In the work now reported a sample of external carcass fat of sheep has been investigated by methods which did not include hydrogenation, and it has been found to contain appreciable quantities of margaric acid.

EXPERIMENTAL

A sample of external carcass fat of sheep (G/99; 31.2 kg.) was steam-rendered, saponified, and converted into fatty acids. The fatty acids were steam-distilled (see McInnes, Hansen & Jessop, 1956), and the steam-non-volatile acids (21.58 kg.) were converted into methyl esters (20.47 kg.) and distilled *in vacuo* in a fractionating column (approx. 180 cm. $\times$ 12 cm.) packed with 0.4–0.2 mm. stainless-steel packing of the type described by Lecky & Ewell (1940). Six fractions and a residue resulted, the fourth fraction (F4; 4285 g., saponification equiv. 288.6, iodine value 51.1, m.p. approx. 15.0°) being relevant to this paper. Fraction F4 was refractionated *in vacuo*, a 490 cm. $\times$ 3.8 cm. stainless-steel column packed with 3–4 mm. diameter single-turn glass helices being used. The first fraction distilled (F4, 1; 376 g., saponification equiv. 276.8, iodine value 18.8) was then refractionated in column G (described by Shorland, Gerson & Hansen, 1955), yielding 11 fractions (F4, 1, 1–F4, 1, 11) and a residue (F4, 1, R; 37.40 g., saponification equiv. 296.1, iodine value 58.8, m.p. 15.5–16.0°). Fraction F4, 1, R (34.7 g.) was then refractionated in column E (Shorland, 1952) into 13 fractions (F4, 1, R1–F4, 1, R13) and a residue (F4, 1, RR; see Table 1).

Fractions F4, 1, R1–F4, 1, R5 were combined, denoted X1, and as acids submitted to a series of low-temperature