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ISOLATION AND IDENTIFICATION OF 17α-OESTRADIOL IN THE BILE OF A PREGNANT COW

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Summary. Free 17α-oestradiol has been isolated and identified in a pregnant cow's bile. The identification was based on several specific colour reactions, the behaviour of different derivatives of the compound in alumina and thin-layer chromatography and finally on a great number of gas chromatographic investigations. A sensitive and highly specific colour test for 17α-oestradiol and its derivatives in thin-layer chromatograms, making use of phosphomolybdic acid, has been presented.

INTRODUCTION

Although 17α-oestradiol has been detected in the urine of several species, including the horse (Wintersteiner & Hirschmann, 1937; Hirschmann & Wintersteiner, 1938), the rabbit (Stroud, 1939; Heard, Bauld & Hoffmann, 1941; Fish & Dorfman, 1942), the cow (Klyne & Wright, 1956a; Velle, 1958a), the goat (Klyne & Wright, 1956b, 1957) and the newborn calf (Velle, 1958b), comparatively little is known about the metabolism of this steroid in the animal organism (Breuer & Pangels, 1960). It has also been found in the placenta of the cow and the ewe (Velle, 1958b) and in the meconium of the calf, lamb and kid (Velle, 1958b).

It is well known that unconjugated oestrone is present in the bile of pregnant cows (Pearlman, Rakoff, Cantarow & Paschkis, 1947) and since 17α-oestradiol is the main urinary oestrogen of this animal during pregnancy, it was thought likely that 17α-oestradiol would also occur in the bile. Moreover it seemed of interest to develop methods for the isolation and identification of microgram amounts of this compound and then renew the search for this oestrogen in the human organism. The present study, therefore, deals with the isolation and identification of 17α-oestradiol in the bile of a pregnant cow and describes a procedure for the identification of very small amounts of this compound in biological extracts.

MATERIAL AND METHODS

The bile was obtained by puncture of the gall-bladder of a cow killed in the 6th month of pregnancy: 400 ml bile was obtained in this way and immediately placed in a deep-freeze until processed.

REAGENTS AND REFERENCE COMPOUNDS

All reagents were of analytical grade unless otherwise stated. The distillations were made through all-glass fractionating columns. The ethyl ether was shaken with $FeSO_4$ solution and distilled water and re-distilled with NaOH pellets.

The hexamethyldisilazane and the trimethylchlorsilane were obtained from Fluka AG, Buchs, Switzerland. For the gas chromatography the following stationary phases were used: 2% QF-1 (fluoroalkyl silicone), 1% SE-30 (dimethyl silicone) and 1% XE-60 (cyanoethyl silicone), which were all obtained from Applied Science Laboratories, Inc. (State College, Pa., U.S.A.), 1% Z (copolymer made from ethylene glycol, succinic acid and a methyl siloxane monomer) and 3% F-60 (methyl-p-chlorophenyl siloxane polymer). The two last-mentioned stationary phases were kindly presented to us by Dr Horning, of Houston, Texas.

The 3-methoxy-oestrone and 3-methoxy-17 β-oestradiol were obtained from Schering AG, Berlin, Western Germany, and the 3-methoxy-oestriol from Sigma Chemical Co., St Louis, U.S.A. All other steroids were supplied by the courtesy of Professor W. Klyne and Professor H. Breuer. The cholestane was obtained from the Aldrich Chemical Co. Inc., Wisconsin, U.S.A.

EXTRACTION PROCESS

The bile sample of 400 ml was extracted in four 100 ml aliquots according to a method which has been described in detail previously (Adlercreutz, 1962). The main steps of this procedure were as follows:

1. Precipitation of fatty material in cold methanol (Zander & Simmer, 1954, as modified by Adlercreutz, 1962). Following centrifugation the precipitate was discarded and the methanolic extracts pooled and evaporated to dryness; the procedure was repeated in 300 ml of 70% (v/v) methanol. Following this second precipitation procedure the methanolic extract was evaporated to dryness.

2. Separation of oestrogen 'sulphates' from oestrogen 'glucosiduronates'. The dry residue of the methanolic extract was dissolved in 100 ml of 0·1 N-sodium hydroxide saturated with sodium chloride and extracted three times with 100 ml of *n*-butanol petroleum ether (70 : 30 v/v) saturated with water and sodium chloride. The combined butanol/petroleum ether extracts ('sulphate fraction') were washed with 10 ml of 0·1 N-sodium hydroxide saturated with sodium chloride.

3. Extraction of the free oestrogens from the 'sulphate' fraction with ethyl ether. Only the free oestrogen fraction was analysed in the present investigation. The ether was washed with Brown's carbonate buffer of pH 10·5 (Brown, 1955) 8% sodium bicarbonate solution and distilled water.

4. A toluene–sodium hydroxide partition. In this way the phenolic steroids were separated from the neutral ones. The sodium hydroxide was then buffered with ammonium sulphate and extracted with ethyl ether, and the ether extracts were washed with 8% sodium bicarbonate and distilled water, and evaporated to dryness.

5. Separation of oestriol from oestrone and oestradiol by solvent partition according to Brown (1955).

6. Methylation of the oestrone-oestradiol fraction and extraction of the methylated oestrogens by *n*-hexane. No hydrogen peroxide oxidation, as used by Brown (1955), was carried out.

7. Chromatography of the methylated oestrogens according to Brown (1955). The methylated oestradiol fraction obtained after chromatography was used for the identification experiments.

PREPARATION OF DERIVATIVES

Methylation. This was carried out exactly as described by Brown (1955).

Acetylation. The acetates of the compounds were prepared by adding 1 ml acetic acid anhydride and 50 µl pyridine to the reference compounds or to the dry residue of the bile extract at room temperature.

Trimethylsilyl ethers. These were prepared according to Luukkainen, Vanden-Heuvel, Haahti & Horning (1961). The compounds were dissolved in 1 ml freshly distilled chloroform or pyridine, 100 µl hexamethyldisilazane and 10 µl trimethylchlorsilane were added, and the glass tube was closed very carefully with a glass stopper and left to stand overnight at room temperature. Following evaporation of the solvent, the residue was dissolved in 1 ml *n*-hexane and centrifuged and the clear supernatant pipetted off into another tube. The precipitate was washed again with 0·5 ml *n*-hexane and centrifuged, and the *n*-hexane solution was added to the previous extract. The *n*-hexane extract was then evaporated under a stream of nitrogen to the desired volume.

THIN-LAYER CHROMATOGRAPHY (TLC)

The original apparatus for thin-layer chromatography manufactured by Desaga, Heidelberg, Western Germany, was used for the preparation of the thin-layer plates. The technique described by Stahl (1959) was followed. The layer of silica gel G was activated at 105° C for 30 min and the plates were then kept in a desiccator at room temperature until used. The chromatograms were run in tanks lined with filter paper in order to obtain complete saturation of the system. Two different solvent systems were used and the running time at room temperature was about 1 hr for both. System A consisted of benzene-absolute ethanol (95 : 5 v/v) and system B consisted of ethyl acetate–cyclohexane (45 : 50 v/v). Following chromatography the zones under investigation were scratched off from the plates and eluted with methanol. The silica gel was centrifuged off and re-eluted with another aliquot of methanol, which, after centrifugation, was added to the first extract. The methanol extract was then evaporated to dryness under a stream of nitrogen.

COLOUR REACTIONS

Kober reaction. This was carried out as described by Nocke (1961).

Extraction of the Kober colour according to Ittrich (1958). This was carried out with chloroform as described previously (Adlercreutz, 1962).

Kägi–Miescher reaction (1939). To the tube containing the dry steroid or bile

compound 1·0 ml glacial acetic acid and 50 µl concentrated sulphuric acid were added, and the tube was placed in a boiling water-bath for 5 min.

Anisaldehyde–sulphuric acid reaction. This was carried out as first described by Stahl & Schorn (1961). In this form it seems not to have been used for the detection of steroids. The reagent is freshly prepared and contains 8·5 ml methanol, 0·5 ml anisaldehyde and 1·0 ml concentrated sulphuric acid. This mixture is sprayed on the TLC plates and the plates are heated at 100° C for 10 to 15 min. The results for the different steroids are seen in Table 1.

TABLE 1

COLOUR OF THE DIFFERENT STEROIDS INVESTIGATED ON THIN-LAYER CHROMATOGRAMS WITH THE PHOSPHOMOLYBDIC ACID AND ANISALDEHYDE-SULPHURIC ACID REACTION

Steroid	*Phosphomolybdic acid*	*Anisaldehyde–sulphuric acid*
Oestrone	Blue→brownish-yellow	Yellowish-brown
3-Methoxy-oestrone	Pale blue	Brown
2,3-Dimethoxy-oestrone	Greyish-blue	Blue
16-Oxo-oestrone	Greyish-blue	Blue
16α-Hydroxy-oestrone	No colour	Reddish-brown
17α-Oestradiol	Dark violet	Bright blue
17α-Oestradiol diacetate	Dark violet	Bright blue
3-Methoxy-17α-oestradiol	Dark violet	Bright blue
3-Methoxy-17α-oestradiol acetate	Dark violet	Bright blue
3-Methoxy-17α-oestradiol-17-trimethylsilyl ether	Dark violet	Bright blue
17β-Oestradiol	Pale blue	Bright green
17β-Oestradiol diacetate	Pale blue	Bright green
3-Methoxy-17β-oestradiol	Pale blue	Dark green
3-Methoxy-17β-oestradiol acetate	Pale blue	Dark green
3-Methoxy-17β-oestradiol-trimethylsilyl ether	Pale blue	Bright green
16-Oxo-17β-oestradiol	Blue→brownish-yellow	Yellowish-brown
2-Methoxy-17β-oestradiol	Blue	Greyish-green
2,3-Methoxy-17β-oestradiol	Greyish-blue	Bluish-green
Oestriol	Blue→brownish-yellow	Brownish-green
16-*Epi*oestriol	Greyish-blue	Pale greyish-violet
17-*Epi*oestriol	Greyish-blue	Pale blue
16,17-*Epi*oestriol	Pale violet	Bluish-grey
3-Methoxy-oestriol	Blue	Bluish-violet
2-Methoxy-oestriol	Bluish-grey	Pale bluish-grey
Testosterone	Pale blue	Dark green
*Epi*testosterone	Blue→yellowish-brown	Blue→black
Pregnenolone	Greyish-blue	Greyish-green
17α-Hydroxy-pregnenolone	Greyish-blue	Greyish-green
Progesterone	Pale blue	Brown
17α-Hydroxy-progesterone	Pale blue	Yellowish-brown
Pregnanediol	Pale blue	Bright blue

Phosphomolybdic acid reaction. Phosphomolybdic acid has been used in a 10% solution for the detection of steroids on paper chromatograms (Kritchevsky & Kirk, 1952). It has also been used in a 2% solution by Lewbart & Schneider (1954) for the detection of conjugated neutral steroids on paper chromatograms. In the present study it was observed that the spraying of the thin-layer chromatograms with a 2% alcoholic solution of phosphomolybdic acid and heating of the plates at 100° C produced a characteristic colour with 17α-oestradiol. The colour, which is very intense, is light pink after 4 to 5 min, then changes to rose, and after 8 min is dark violet. None of the steroids listed in Table 1 gives this

colour within that time, except for the different derivatives of 17α-oestradiol. After 10 min the other steroids usually give other blue colours, with the exception of 16,17-*epi*oestriol, which gives a pale violet colour. Since 17-*epi*oestriol gives a greyish-blue colour, it seems likely that a hydroxyl in the 16 α-position hinders the development of the violet colour found to be specific for the 17α-hydroxyl in 17α-oestradiol. The reaction is very sensitive and can be detected if the steroid is present in amounts of 0·1 to 0·2 μg. It is interesting to note that methylation at C-3 and the trimethylsilyl ether derivative or acetate of the 17α-oestradiol give the same dark violet colour. This reaction seems to be very useful when attempts are made to isolate 17α-oestradiol from biological material.

GAS CHROMATOGRAPHY

Two types of gas-chromatography apparatus were used in this work. A Chromalab model 110 (Glowall Corporation Pennsylvania, U.S.A.) and a Barber Colman model 15 (Barber-Colman Co., Wheelco Industrial Instruments, Rockford, Illinois, U.S.A.), both equipped with a Lovelock argon ionization detector containing a radium foil source. In every analysis the instruments were isothermal. All liquid phases were on 100 to 140 mesh Gas-Chrom P. The oven of the Chromalab contained a 6 ft × 4 mm coiled glass column and that of the Barber Colman a 6 ft × 9 mm U-shaped glass column. The columns were prepared according to methods developed earlier for analytical work with steroids (VandenHeuvel & Horning, 1962). The samples (1 to 2 μl) were introduced with a Hamilton micro-syringe.

It is necessary to test the solvents used in the gas chromatograph. Therefore, in the present investigation, after evaporation of an aliquot of 10 to 50 ml of every solvent, the residue of the solvent and its trimethylsilyl and acetyl derivatives were tested on the gas chromatographic columns used, in order to find out whether any impurities were present. It was found that the residue of ethyl acetate gave a peak after conversion to trimethylsilyl derivatives. This was eliminated by redistillation. The trimethylsilyl derivatives of the evaporated methanolic extract of silica gel G gave several minute peaks; if the concentration of steroids was very low in such a sample it was necessary to run a parallel gas chromatogram of a similar extract of thin layer silica gel.

The reference solution of the di-trimethylsilyl ether of 17α-oestradiol was employed at different concentrations to estimate the quantity of steroid in the samples. For the determination the areas of the peaks were calculated from their heights and widths at half-height. It was found that the response was linear from zero to 10 μg.

RESULTS

A small part (1/8th) of the methylated and chromatographed compound obtained from the cow's bile was submitted to the Kober reaction. It was found that the total bile sample contained 24 μg of a Kober-positive compound resembling 3-methoxy-17α-oestradiol in its spectral characteristics. The absorption maximum was 524 mμ, which was the same as was found for authentic 3-methoxy-17α-oestradiol. Following Ittrich extraction of the Kober

colour the absorption maximum of the compound shifted to 538 mμ both for the bile compound and the authentic 3-methoxy-17α-oestradiol reference standard.

Another sample (1/8th) was submitted to the Kägi–Miescher reaction, which is said to be specific for 17α-oestradiol. It was found that the characteristic pink colour developed for both the bile compound and the authentic reference standard of 3-methoxy-17α-oestradiol.

A small amount of the isolated and methylated compound (Fraction I in Table 2) was investigated with gas chromatography on the F-60 column.

TABLE 2

GAS CHROMATOGRAPHIC INVESTIGATION OF DIFFERENT FRACTIONS OF THE 17α-OESTRADIOL-LIKE COMPOUND OBTAINED FROM PREGNANT COW'S BILE ON FIVE DIFFERENT LIQUID PHASES

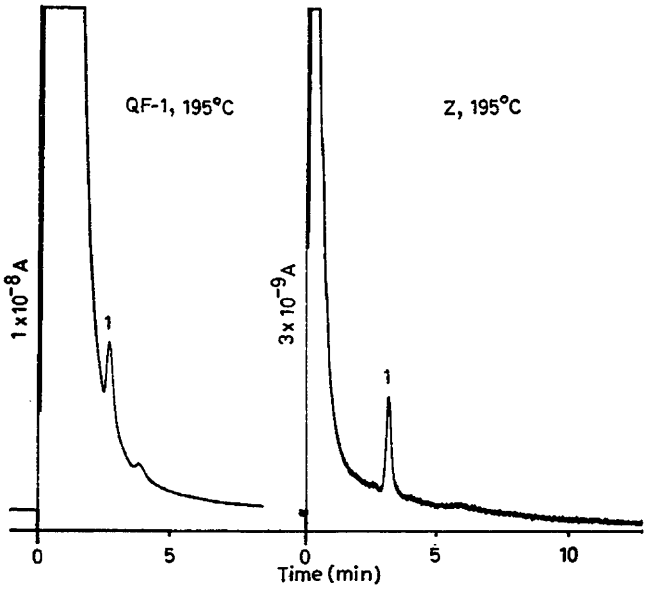
Fraction	*Relative retention time*													
	2% *QF*-1*				1% *XE*-60*				3% *F*-60*		1% *Z**		1% *SE*-30*	
	194° *C*		208° *C*		195° *C*		205° *C*		227° *C*		195° *C*		227° *C*	
	Bc†	*Rc*	*Bc*	*Rc*	*Bc*	*Rc*	*Bc*	*Rc*	*Bc*	*Rc*	*Bc*	*Rc*	*Bc*	*Rc*
I									0·53	0·54				
II	0·67	0·66					0·67	0·67	0·50	0·51	1·13	1·11	0·51	0·51
III	0·68	0·66			0·68	0·68	0·68	0·68	0·51	0·52	1·11	1·11		
IV							1·37	1·39						
V			1·42	1·38			1·37	1·37	0·67	0·68	4·14	4·16		
VI					0·69	0·68	0·70	0·68	0·52	0·52	1·09	1·11		

The different fractions are described in the text. Fraction I = methylated compound. Fractions II, III and VI = trimethylsilyl ether derivative of the methylated compound. Fractions IV and V methylated and acetylated compound. Argon inlet pressure 2 kg/cm² for all columns with the exception for the SE-30 column (1·5 kg/cm²). All liquid phases on 100 to 140 mesh Gas-Chrom P.A Chromalab model 110 and a Barber Colman model 15 (only for the SE-30 column) were used.

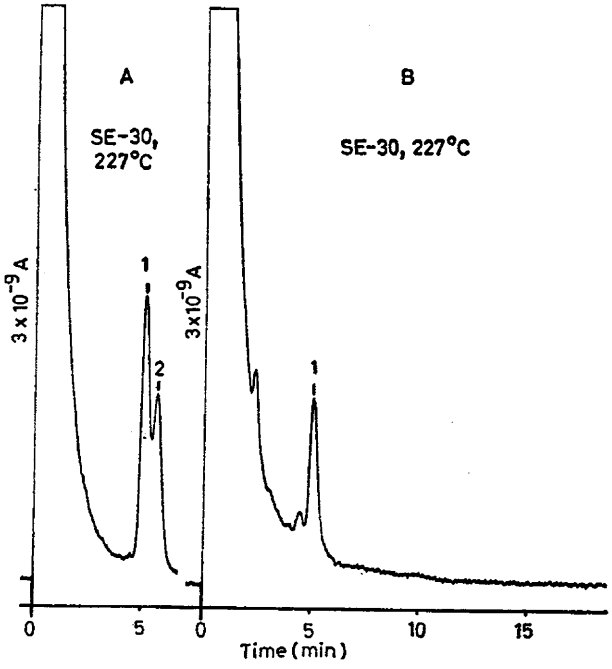
* Liquid phase.
† Bc = bile compound; Rc = reference compound.

A peak corresponding to 3-methoxy-17α-oestradiol was found. Since the methylated 17α-oestradiol and 17 β-oestradiol do not separate to any satisfactory degree on any column, no further investigations were carried out with the methylated compound as such.

A part of the remaining sample was then converted to the trimethylsilyl ether derivative and this compound (Fraction II in Table 2) was investigated on five different liquid phases, since now a good separation from the corresponding 17β-oestradiol derivative could be achieved. All gas chromatograms are shown in Text-figs. 1 to 3 and in two of the figures (Text-figs. 2A and 3A) the separation of the 3-methoxy-17α-oestradiol-17-trimethylsilyl ether from the 3-methoxy-17β-oestradiol-17-trimethylsilyl ether is demonstrated. In none of the gas chromatograms could any evidence indicating the presence of 17β-oestradiol in the cow's bile be obtained. It could therefore be concluded that no free 17β-oestradiol is present in the pregnant cow's bile or that its concentration is extremely low. It can also be seen from Text-figs. 1 to 3 that the methylated



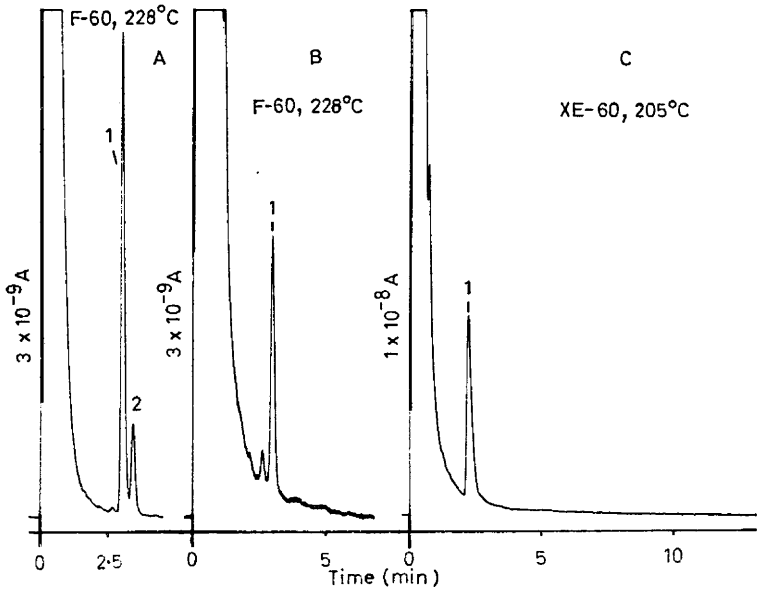
TEXT-FIG. 1. Gas chromatography of the trimethylsilyl ether derivative of the methylated 17α-oestradiol-like compound (peak 1) obtained from pregnant cow's bile on two different stationary liquid phases. Chromalab model 110, argon inlet pressure 2 kg/cm².



TEXT-FIG. 2. Gas chromatography of the trimethylsilyl ether derivative of the methylated 17α-oestradiol-like compound (peak 1 in B) obtained from pregnant cow's bile and of the same derivatives of the reference compounds of 17α-oestradiol (peak 1 in A) and of 17β-oestradiol (peak 2 in A) on the SE-30 stationary phase. Barber Colman model 15, argon inlet pressure 1·5 kg/cm².

fraction obtained from the cow's bile was very pure, only traces of impurities being present.

The rest of the bile compound was then submitted to TLC in system A. The zone corresponding to methylated 17α-oestradiol was localized with the anisaldehyde reaction and was found to have an R_f-value of 0·25. The 3-methoxy-17α-oestradiol reference compound had R_f-value of 0·24. The zone corresponding to R_f 0·2 to 0·3 was scratched from the plate and a part of the fraction (1/3rd) was converted to its trimethylsilyl ether derivative (Fraction III in Table 2) and run on four different gas chromatographic columns. The relative retention



TEXT-FIG. 3. Gas chromatography of the trimethylsilyl ether derivative of the methylated 17α-oestradiol-like compound (peak 1 in B and C) obtained from pregnant cow's bile on two different liquid phases and of the same derivatives of the reference compounds of 17α-oestradiol (peak 1 in A) and of 17β-oestradiol (peak 2 in A) on one stationary phase. Chromalab model 110, argon inlet pressure 2 kg/cm².

times of the bile compound and the corresponding reference compound are seen in Table 2, and there is satisfactory agreement between them.

Part of the fraction chromatographed in TLC system A (1/3rd) was acetylated and a very small amount of this compound (Fraction IV in Table 2) was investigated on the XE-60 column. It was found that the methylated and acetylated compound obtained from the cow's bile had a retention time identical with that of authentic 3-methoxyl-17α-oestradiol-17-acetate. The rest of the acetylated fraction was then chromatographed in TLC system B and the zone corresponding to the methylated and acetylated 17α-oestradiol was localized with the phosphomolybdic acid reaction. The R_f of the zone was 0·61 and that of the authentic reference standard the same. The zone corresponding to R_f 0·59 to 0·67 was scratched from the plate and eluted with methanol and following evaporation the compound (Fraction V in Table 2) was submitted to gas chromatographic analysis on four different columns. The results are shown

in Table 2 and it will be seen that there is satisfactory agreement between the methylated and acetylated compound obtained from the cow's bile and the reference standard on all the columns used.

The last part of the methylated bile compound chromatographed in TLC system A was submitted to TLC in system B and the zone corresponding to 3-methoxy-17α-oestradiol was localized with the phosphomolybdic acid reaction. The bile compound had an R_f-value of 0·52, the corresponding reference compound an R_f-value of 0·51. The zone corresponding to R_f 0·48 to 0·56 was scratched from the plate, eluted and rechromatographed on partially deactivated alumina (Brown, 1955) and converted to its trimethylsilyl ether derivative. This fraction (Fraction VI in Table 2) was submitted to gas chromatographic analysis on three different columns (Table 2). The results indicate that there is good agreement between the results obtained with the bile compound and with the reference standard.

The traces of all samples of trimethylsilyl ether derivative of the methylated bile compound were finally chromatographed in the TLC system B and the spot was developed with the phosphomolybdic acid reaction. The colour of the spot (dark violet) agrees completely with that of the trimethylsilyl ether of the 3-methoxy-17α-oestradiol reference compound. The R_f-value of both compounds was 0·70.

Thus the identification of free 17α-oestradiol in the pregnant cow's bile is based on the following evidence:

1. The characteristic colour of the derivatives of the compound in four different colour reactions.
2. The absorption spectrum of the methylated compound in the Kober reaction and subsequent extraction of the Kober colour according to Ittrich.
3. The chromatographic behaviour of the methylated compound on partially deactivated alumina and in two TLC systems.
4. The chromatographic behaviour of the methylated and acetylated compound in one TLC system and of the trimethylsilyl ether derivative of the methylated compound in one TLC system.
5. The gas chromatographic behaviour of the methylated compound, the methylated and acetylated compound and the trimethylsilyl ether derivative of the methylated compound in altogether twenty different gas chromatograms on five different liquid phases and sometimes using different temperatures for the same stationary phase.

In view of the accumulated evidence it was therefore concluded that the isolated compound was indeed identical with 17α-oestradiol.

DISCUSSION

The colour reactions are more or less unspecific. When it was found that among oestrogens 17α-oestradiol gave a specific colour with phosphomolybdic acid, it was assumed that the reaction would be the same with all 17α-hydroxysteroids. However, the other 17α-hydroxysteroids studied, including 17-*epi*testosterone, did not react in the same way. Therefore in addition to the 17α-hydroxy group an aromatic A ring of the steroid nucleus is needed, which renders the phosphomolybdic acid reaction described surprisingly highly specific for 17α-oestradiol.

The use of several thin-layer chromatogram systems with sensitive localization of 17α-oestradiol combined with the utilization of the high separation capacities of many gas chromatographic liquid phases makes it possible to isolate and identify extremely small amounts of 17α-oestradiol in biological material.

The conversion of the compound under investigation to different derivatives and gas chromatographic analysis of these on several liquid phases is essential for the reliability of the identification.

Pearlman *et al.* (1947) isolated oestrone from pregnant cow's bile. It was present in amounts of approximately 600 μg/l of bile. They further observed that the oestrogenic activity of the non-ketonic, weakly acidic phenolic fraction in bioassay was equivalent to 70 μg/l of α-oestradiol (17β-oestradiol) and most of the oestrogenic material of bile was present in free form. The present investigation confirms their results. Although not mentioned earlier, free oestrone was also found in the cow's bile, but no evidence indicating the presence of oestriol. The amount of 17α-oestradiol was the same, 6 μg/100 ml of bile in this stage of pregnancy (6th month), as estimated both with the Kober reaction and quantitative gas chromatography. The estimated oestrogenic activity of the diol fraction found by Pearlman *et al.* (1947) seems to be due solely to 17α-oestradiol, because no evidence of the presence of free 17β-oestradiol was found in the present study.

The method developed is now being used in the search for 17α-oestradiol in the human organism. Some evidence has already been obtained indicating that this compound may possibly be present in human bile in late pregnancy (Adlercreutz & Luukkainen, 1965). However, the amounts present are so extremely small that isolation and definite identification are very difficult.

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