THE STEROID SULPHATES

Studies on the Conjugated Sulphates of Mare's Pregnancy Urine.

by

WILLIAM KLYNE
M.A., B.Sc.(Oxon.)

A Thesis presented for the Degree of Doctor of Philosophy in the University of Edinburgh.

November 1946.

Contents.

INTRODUCTION and SUMMARY.

The original purpose of this work was to determine the constitution of an organic half-ester of sulphuric acid isolated from mare's pregnancy urine by Schachter and Marrian in Toronto in 1938.

Two new organic sulphates, one of which may be identical with that of Schachter, have been isolated and studied. The scope of the work has been widened, so that it now represents some initial stages in a general study of the conjugates of horse urine. Some incidental work on the pure chemistry of organic sulphates has also been done.

Part 1 of this Thesis outlines the previous work on the organic sulphates of mare's pregnancy urine (Schachter and Marrian, 1936, 1938; Schachter, Ph.D. Thesis, 1939; Butenandt and Hofstetter, 1939).

Part 2 describes the sources and collection of urine, the extraction of the sulphates and the early stages in their purification. These methods have been developed from those of Schachter.

Part 3 describes the isolation and study of a sulphate (Z sulphate) which may be identical with that/

that of Schachter and Marrian. This sulphate on acid hydrolysis yields a compound of the probable formula $C_{21}H_{32}O_2$ (Compound Z) which is apparently not identical with any substance previously obtained from mare's pregnancy urine. Z appears to be a 3-(β)-hydroxy steroid and may contain an αβ-unsaturated ketonic group. Comparison of Z and Δ^{16} allopregnene-3(β)-ol-20-one indicates that these substances are probably identical, although the evidence is insufficient for proof.

Part 4 describes the isolation and study of another sulphate (Y sulphate). This on acid hydrolysis yields Compound Y, probable formula $C_{21}H_{36}O_2$. Y is a saturated dihydroxy compound, probably a 3(β)-hydroxy steroid. The properties of Y and its derivatives closely resemble those of 'uranediol' and its derivatives (Marker et al., 1938f), but no direct comparison has yet been possible.

Part 5 of this Thesis describes miscellaneous experiments on mare's urine conjugates, incidental to the study of Z and Y, which may be useful if a thorough study of horse-urine steroids is undertaken.

The great drawback throughout the work has been lack of material. Probably more time has been spent in looking for urine than in working it up. So far as the primary purpose of the work is concerned, lack/

lack of material has prevented a complete study of Compounds Z and Y. In the wider field, few definite results have been obtained; it has rarely been possible to study a number of batches of urine in the same way, and so to obtain results of quantitative significance. Again, while many different methods of extraction and separation have been tried at different times (Part 2, section 6), controls have rarely been carried out, and this part of the work must be considered a very sporadic reconnaisance. The author begs anyone who reads this thesis to bear these facts constantly in mind.

This work has been supported by grants from the Agricultural Research Council. It is hoped that it may some day be possible to continue the work on broader lines. If adequate supplies of urine are assured, the author is confident that many interesting and probably useful results can be obtained. An outline of the author's suggestions for further work is given in Appendix A.

If, however, it is impossible to obtain supplies of urine sufficient to get quantitatively significant results and to carry out controls in developing practical methods, the work should cease (apart from further attempts to determine the constitution/

constitution of Z and Y).

The work has made desirable studies on the synthesis and properties of organic sulphates (especially steroid sulphates). These studies will be continued, whether or not the urine problem is to be attacked further.

The literature on the steroids of horse urine is summarised in Appendix B. Appendix C gives some notes on 17-isopregnane derivatives, which may be of interest in connection with the structure of Compound Y. Literature references (in the form used in the Biochemical Journal) are collected alphabetically in Appendix D.

I wish to thank the following for their help:

My supervisor, Professor G.F. Marrian, F.R.S., for suggesting this problem and for his constant advice and interest.

Alexander Purdie, laboratory assistant, for much skilful and willing help in stable and laboratory.

The Agricultural Research Council and the Moray Fund of this University for grants.

The Ovaltine Research Laboratories (Dr F.Wokes), Messrs British Drug Houses Ltd. (Dr P.G.Marshall) and the Animal Diseases Research Institute, Moredun, Edinburgh (Dr J. Russell Greig, C.B.E.) for supplies of urine.

Mrs G.F.Marrian and Mr J.W. Minnis for micro-analyses.

Messrs Goodlass Wall and Lead Industries, Ltd. (Mr C.A.Klein, the author's father) for the loan of large scale apparatus and for the gift of high-grade lead peroxide for micro-analysis.

Professor/

Professor T. Reichstein (Basel) and Professor Pl.A. Plattner (Zürich) for authentic samples of Δ^{16}-allopregnene-3(β)-ol-20-one. Further to Professor Reichstein for mixed melting point determinations and for advice on several points.

Professor J.W. Cook, F.R.S. (Glasgow) for two micro hydrogenations done in his Department.

Mr J. Rankeillor, Senior Technician, for the design and construction of urine collecting apparatus and large separator.

PART 1.

PREVIOUS WORK ON THE CONJUGATED SULPHATES OF MARE'S PREGNANCY URINE.

1, 1. PURPOSE OF SCHACHTER'S WORK.

Schachter and Marrian (1936, 1938; for details see Schachter, Ph.D. Thesis, 1939) studied the conjugated oestrogens of mare's pregnancy urine; from this source they obtained with much difficulty potassium oestrone sulphate and another substance which appeared to be a steroid sulphate, referred to here as 'A sulphate'.

1, 2. SCHACHTER'S METHOD FOR THE ISOLATION OF A SULPHATE.

Mare's pregnancy urine was extracted with butyl alcohol and acidic material was removed from the butyl alcohol extracts. The neutral material from the butyl alcohol extracts was partitioned between chloroform and water. The materials which passed into aqueous solution were treated with barium acetate and the precipitated barium salts reconverted to their potassium salts. (Details of these stages are given in Part 2, section 6 of this thesis, since they formed the basis for our methods). The potassium salts were dissolved in acetone containing a trace of water, and on evaporation of most of the acetone, crude potassium A sulphate separated, usually as white needles (m.p. between 200° and 214°). Potassium oestrone sulphate was obtained with great difficulty from the mother liquors after the removal of A sulphate. Potassium A sulphate was purified by recrystallisation from aqueous acetone.

1, 3. EXCRETION OF POTASSIUM A SULPHATE.

The quantities of crude potassium A sulphate isolated by Schachter from different batches of urine are given below (Table 1). It is not known whether all these batches of urine were from the same mare.

Table 1.

Period of pregnancy months	Mg. of $KASO_4$ isolated per litre of urine
4	2
6	10
6¼	12
7¾	22
8	23
8	18
8¼	41

1, 4. PROPERTIES OF POTASSIUM A SULPHATE.

White needles from aqueous acetone. M.p. 215.5-216.5°. Soluble in hot water, much less soluble in cold. Soluble in aqueous acetone, methyl alcohol and ethyl alcohol. Insoluble in anhydrous acetone, ether, benzene and chloroform.

Contained sulphur, but no nitrogen or halogens. Heating with dilute hydrochloric acid liberated sulphate ions. Contained no glucuronic acid.

Analyses. No analyses for $KASO_4$ were published since the results were inconsistent. The following figures were obtained from Schachter (private communication).

Found/

Found:	%C	%H	%S	%K
Sample 1 (a)	55.49	8.57		
2	56.78	8.78	5.53	5.00(b)
3	56.83	8.72	5.39	
4	56.62	8.76	5.60	6.62

(a) This sample was suspended in water and evaporated to dryness on the water bath under nitrogen to remove possible acetone of crystallisation; m.p. 198.

(b) Sample probably too small for accurate analysis.

Calculated:	%C	%H	%S	%K
$C_{18}H_{29}O.SO_4K$	54.6	7.3	8.1	9.8
$C_{19}H_{31}O.SO_4K$	55.6	7.55	7.8	9.5
$C_{21}H_{35}O.SO_4K$	57.7	8.0	7.3	8.9

Schachter's hydrolysate (Compound A) gives analytical figures which suggested to me that it might be a saturated diol, $C_{18}H_{30}O_2$, $C_{19}H_{32}O_2$, or $C_{21}H_{36}O_2$. The calculated figures for sulphates of such compounds obviously do not agree with Schachter's figures for $KASO_4$. Schachter states (Thesis, p.63): "It was believed probable that the material consisted of two very closely related compounds in a slightly varying ratio, or that an almost constant impurity was present which could not be removed by the method of recrystallisation employed."

1, 5. /

1, 5. HYDROLYSIS OF POTASSIUM A SULPHATE AND PROPERTIES OF COMPOUND A.

$KASO_4$ was unchanged on treatment with hot 2N aqueous potassium hydroxide. 0.33 N Hydrochloric acid at room temperature precipitated a water-soluble complex (perhaps $HASO_4$); $KASO_4$ is therefore much more stable to acid than is oestrone sulphate.

Hydrolysis was finally achieved with aqueous alcoholic hydrochloric acid. The ether-soluble hydrolysate, Compound A, was purified by recrystallisation from aqueous acetone and then benzene.

Hydrolysate Compound A. Needles from aqueous acetone M.p. 190-192°. Soluble in ether, insoluble in water.

Analyses of A (Schachter, private communication)

Found:	%C	%H
Sample 1	(77.54	11.33
	(77.16	11.25
3	74.57	11.03

The two concordant results on sample roughly agree with the figures for a saturated diol (W.K.)

Calculated:	%C	%H
$C_{18}H_{30}O_2$ (oestranediol)	77.7	10.8
$C_{19}H_{32}O_2$ (androstanediol)	78.1	10.9
$C_{21}H_{36}O_2$ (pregnanediol)	78.8	11.2

No known androstanediol or oestranediol melts in the region of 180-190, but two pregnanediols do, viz:-

Pregnane-3(β)-20(α)-diol	m.p. 182
Allopregnane-3(β)-20(β)-diol	m.p. 192-194

Qualitative/

Qualitative Tests on Compound A.

Test	Result	Conclusion
Millon	Negative	No phenolic OH
Zimmermann	"	No $CO.CH_2$
Tetranitro-methane	"	No olefinic double bond (a)
Conc. H_2SO_4	Usual range of colours for steroids	
Voss	Negative	Not a known oestrogen
Digitonin	ppt., soluble on warming.	Probably 3(β)-OH group

(a) Ruzicka et al. (1929) state that αβ unsaturated acids, carbonyl compounds, or alcohols give no coloration, or a very slowly-forming yellow coloration, with this reagent. See also Hurd (1938).

1, 6. SCHACHTER'S COMPOUNDS B and C.

Schachter also obtained by the same methods two other compounds, potassium B sulphate and potassium C sulphate. The only data available on these compounds are the following (private communication).

Potassium B sulphate, m.p. 224-225°, is excreted chiefly during the last 50 days of pregnancy, when it may appear in quantities up to 2 g. per 24 hours.

Potassium C sulphate, m.p. 240-250°, is excreted chiefly/

chiefly during the period from 200 to 150 days before foaling.

From what we now know about the unreliability of potassium salt melting-points for characterising sulphates (see Part 3, section 3), it seems possible that two or more of Schachter's sulphates were polymorphic modifications of the same substance.

1, 7. WORK OF BUTENANDT AND HOFSTETTER

Butenandt and Hofstetter (1939; details in Hofstetter 1939 (Dissertation, not seen)) were studying the water-soluble conjugated oestrogens of human and mare's pregnancy urine when Schachter and Marrian (1939) announced the isolation of potassium oestrone sulphate. The German workers had synthesised sodium oestrone sulphate and other salts to gain information about their properties which might help in their isolation from natural sources. They had done some work towards the isolation of oestrone sulphate from urine, which was stopped when the paper of Schachter and Marrian was published. Butenandt and Hofstetter, whose paper gives only brief notes on their work with urine, state that the water-soluble conjugated oestrogens of human and mare's pregnancy urine/

urine behave in all ways like synthetic oestrone sulphate; their final product contained 50-75% oestrone sulphate and was, they considered, a mixture of oestrogen sulphates.

The following practical points may be noted:- (i) use of alkaloidal salts of the sulphates for purification, (ii) use of aluminium oxide chromatograms for sulphates, (iii) the presence of indoxyl sulphate, which accompanied oestrone sulphate in the early stages of isolation, but could be separated by its greater solubility in organic solvents. (If normal conditions return in Europe, it would be worth getting a copy of Hofstetter's Dissertation to obtain further information on his methods with urine).

PART 2.

COLLECTION, SOURCES AND WORKING-UP OF URINE.

2, 1. COLLECTION OF URINE.

A simple apparatus for collecting urine from the Department's own mare (Figs. 1 and 2) was designed and constructed by Mr J. Rankeillor. The mare was tethered at the head in a narrow pen, so that she stood with her hind legs on the collecting grid A and could not fail to discharge into the tray C below the grid the greater part of the urine voided. She was given one hour's exercise a day, and for a day a week she was not tethered. She was in excellent condition when she foaled and the tethering (two periods of 5 and 3 weeks respectively) apparently had no ill effects.

The cast-iron grid A (such as is found in pavements to allow light to reach cellar windows; 30 ins. by 40 ins. $1\frac{5}{8}$ ins. thick), was supported on legs BB ($7\frac{1}{2}$ ins. high) at two corners. A galvanised iron tray C to collect the urine was placed beneath A, covering the whole area under it. To minimise contamination of the urine with faeces a wire grid D ($\frac{1}{8}$ in. mesh) was placed between A and C. To minimise the noise made by the animal's rear hooves (H) on the grid A, a little straw was spread over this.

Methods used for the collection of urine elsewhere include the metabolism cage (very expensive) and the collecting-bag; this is a leather or canvas bag with an opening to fit over the mare's vulva, and/

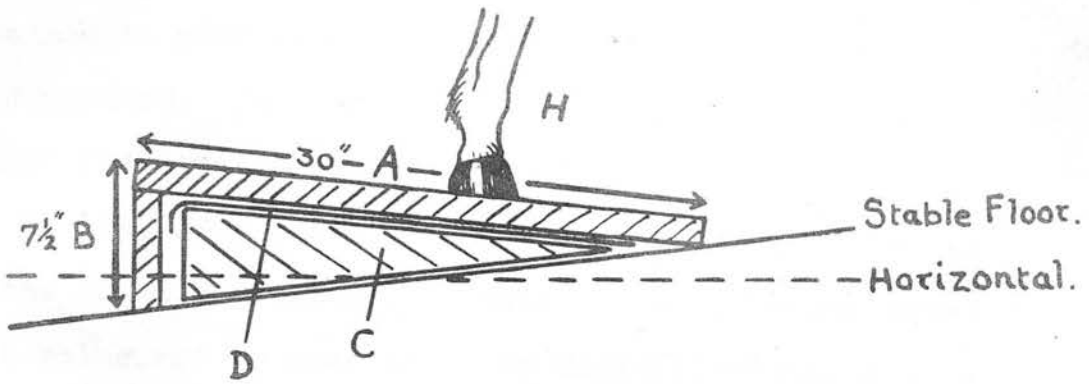


Fig. 1. COLLECTING APPARATUS
Side View

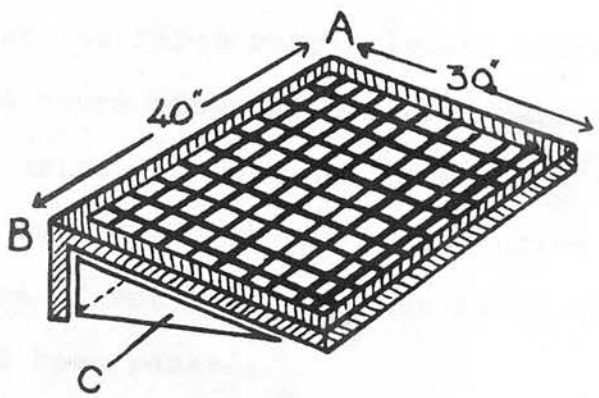


Fig. 2. COLLECTING APPARATUS
Perspective

and an outlet tube leading to a bucket. The bag is retained in position by a harness round the mare's hindquarters. This apparatus has the advantage that a 100% yield of urine, not contaminated by faeces, may be obtained, while the animal need not be tethered. It has the disadvantage, however, that mares are somewhat reluctant to wear it. Mr Rankeillor's apparatus is the cheapest and simplest that we have yet encountered.

2, 2. PRESERVATION OF URINE.

Urine from our own mare was collected every day and given its first butyl alcohol extraction not more than 24 hours after it had been passed.

When urine was obtained from outside sources the senders were asked to add to the urine one tenth of its volume of butyl alcohol not later than 24 hours after it had been passed.

It is not known whether this treatment prevents bacterial decomposition of the desired constituents of the urine, but until more is known about these substances some arbitrary method of preservation such as the above is all that can be attempted.

2, 3 /

2, 3. SOURCES OF URINE.

Source M. Animal Diseases Research Institute, Moredun, Gilmerton, Edinburgh.
4 Shetland ponies.

Batch	Weeks before foaling	Volume (litres)
M 1-2	16	27.4
M 3	15	11.6
M 4	15	6.6
M 5	12	12.2
M 6-7	10-11	12.2
M 8	9	12.7
M 9	8	9.2
M 10	7	7.8
M 11	6	14.6
M 12	3-4	53.1

Source N. Own mare, NANNY
Pony; 12 hands

N 1	13	6.6
N 5	12	8.0
N 11	11	11.2
N 24	8-9	14.7
N 36	0-3	22.2

Source O. Ovaltine Research Laboratories, King's Langley, Herts.
3 Percheron mares.

O1-3	11-14	52.0
O4-5	9-10	35.6
O 6	8	18.0
O 7	6	17.0
O 8	4	16.5
O 9	2	17.5
O 10	1	17.0
O 11	1	18.0
O 12	1	18.5

Source B Messrs British Drug Houses Ltd.

B 13	(Shortly before foaling)	200

2, 4. METHODS FOR LARGE-SCALE EXTRACTIONS.

Extractions were done in 5 l. separating funnels in the usual way, or in 5 or 10 gallon galvanised steel drums kindly lent to us by Messrs Goodlass Wall and Lead Industries Ltd. (through the author's father, Mr C.A.Klein, Technical Director). The drums (see Fig. 3 for dimensions of 10 gallon drum), which were strong enough for use as receivers in distillation at 60-100 mm. pressure, were used for extractions as follows:-

The necessary liquids were poured into the drum, the cap was screwed on tightly and the drum was laid on its side and shaken vigorously to and fro (usually 100 times). The drum was then stood upright, the two layers allowed to separate and the transfer adapter (Fig. 4) inserted. The narrow tube A was connected to the compressed air-line, and the lower liquid layer blown out of the drum through the wide tube B. If it was known that this liquid would have separated sharply, it was blown directly into another can for further extraction or rejection.

If it was suspected that emulsion would be present, the lower layer was blown into the 20-litre separator (Fig. 5). This separator (designed by Mr Rankeillor) consisted of a heavy walled glass bottle in/

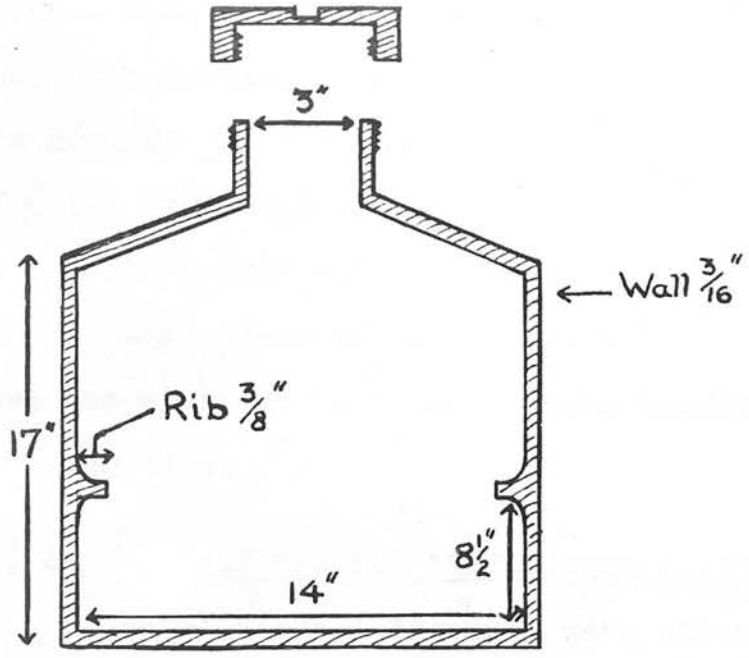


Fig. 3. EXTRACTION DRUM
Capacity - 10 gallons

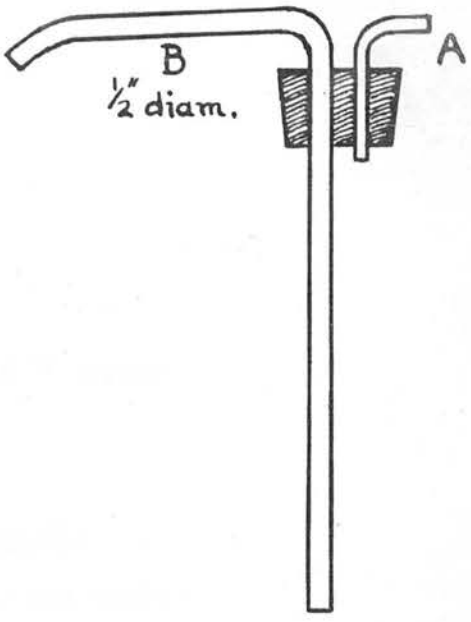


Fig.4. TRANSFER ADAPTER

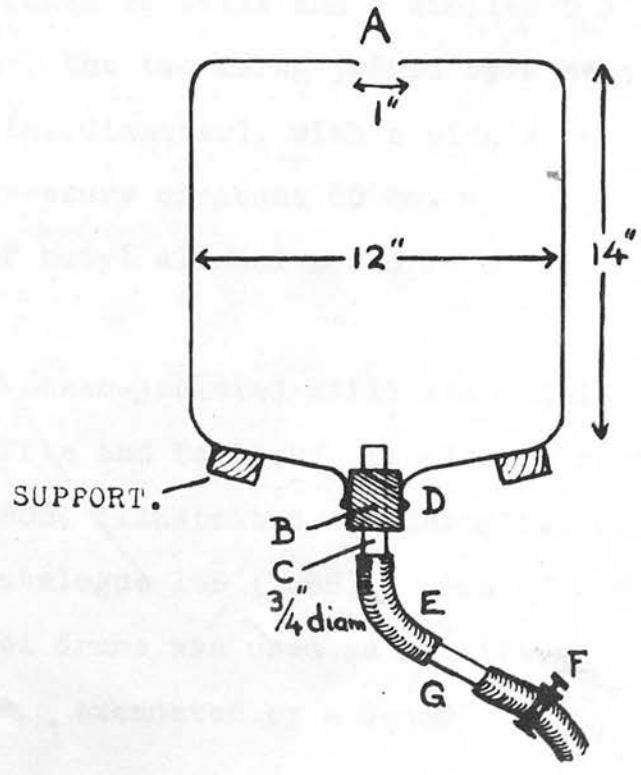


Fig. 5. SEPARATOR
Capacity - 20 litr

in the base of which a hole A (1 in. in diameter) had been drilled. The bottle was inverted (as shown in Fig. 5) and a rubber bung B carrying a glass tube C ($\frac{3}{4}$ in. diameter) inserted into the neck D. The tube C carried a long wide rubber tube E with a screw clip F. A short piece of glass tube G made it possible to see the meniscus as it moved down towards F during a separation.

2, 5. METHODS FOR LARGE SCALE DISTILLATION.

Butyl alcohol extracts were distilled under reduced pressure either in a glass still or a tin-lined steam-jacketed 10 gallon still.

The glass still consisted of a 10 litre Pyrex bolt-head flask as still and a similar 5 litre flask as receiver, the two being joined by a bent glass tube ($\frac{5}{8}$ in. diameter), with a single water pump giving a pressure of about 60 mm. mercury. About 3 litres of butyl alcohol could be distilled per hour.

The steam-jacketed still was obtained from Messrs Griffin and Tatlock; it closely resembled Item B.46-805, illustrated and described on p. 656 of their catalogue 15B (1938). One of our own 10-gallon steel drums was used as receiver. The apparatus was exhausted by a Cenco Hyvac pump, the pressure/

pressure being reduced to about 60 mm. Hg in 25 minutes. The Hyvac pump was then replaced by a single water-pump (to avoid the possibility of contaminating the oil-pump with butyl alcohol vapour) and a vacuum of 60-100 mm. Hg was maintained.

This apparatus distilled about 8 litres butyl alcohol per hour; there was never any sign of bumping or splashing.

2, 6. DEVELOPMENT OF METHODS FOR WORKING UP OF URINE.

This section outlines attempts to improve on Schachter's method, which, it must be remembered was planned primarily to obtain oestrone sulphate and not A sulphate.

The method used for the first few batches of urine in Edinburgh, which is an almost exact copy of Schachter's method, is shown in flow sheet form in Tables 2 and 3. Table 2 covers the original extraction and removal of acidic material; these stages were retained throughout the work. Table 3 covers the chloroform partition and an acidification treatment, which have been discarded.

Our modifications are then listed roughly in chronological order to indicate the development of our ideas. It must be emphasised that, owing to lack /

lack of material, control experiments to compare different methods have rarely been possible. In view of the known large variations in the composition of mare's urine from batch to batch and from one stage of pregnancy to another, these results have little value in themselves. They have, however, helped in the preparation of plans for a thorough study of mare's urine if ever adequate supplies of material are available.

Table 2.

Original Extraction Method (First Part)

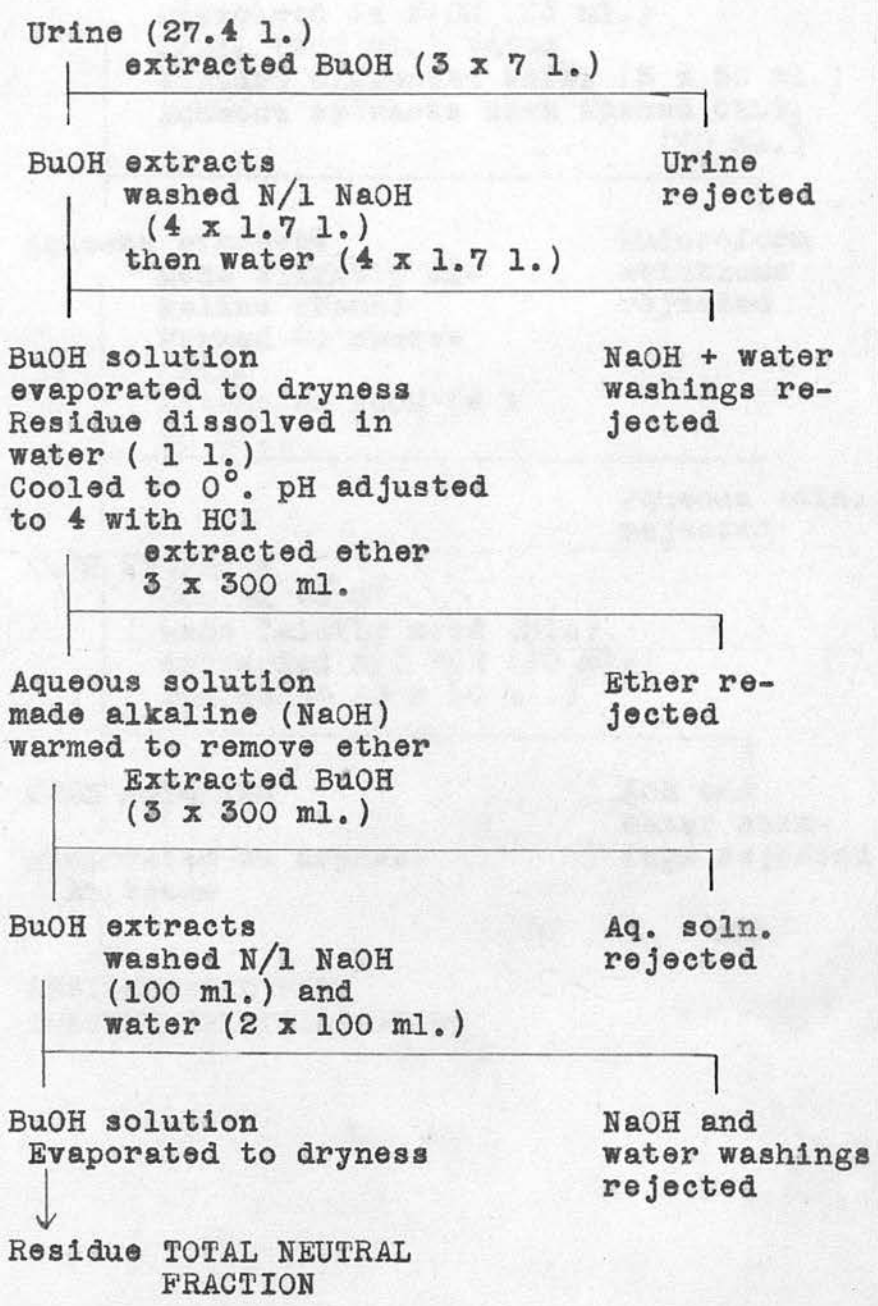


Table 3

Original Extraction Method (Second Part)

Total Neutral Fraction
Dissolved in EtOH (25 ml.)
$CHCl_3$ (300 ml.) added
Mixture extracted water (5 x 50 ml.)
Aqueous extracts back washed $CHCl_3$ (70 ml.)

Aqueous extracts
Made slightly alkaline (NaOH)
Warmed to remove $CHCl_3$
Extracted BuOH (4 x 80 ml.)

Chloroform solutions rejected

Aqueous soln. rejected

BuOH Extracts
Cooled to 0°
Made faintly acid (HCl)
Extracted N/1 KOH (50 ml.)
and water (3 x 50 ml.)

BuOH Solution
Evaporated to dryness in vacuo

KOH and water washings rejected

↓

RESIDUE USED FOR ACETONE CRYSTALLISATION

Batches M1-2, M4, M6-7.

The method of Tables 2 and 3 was used. Very small quantities of material resembling Schachter's sulphates were obtained. Water crystallisation of material from M1 ultimately gave some Z sulphate.

MODIFICATIONS

Batch M3.

Idea - The precipitation of the barium salts, used by Schachter as a stage in the purification of oestrone sulphate, had been omitted in our original method. It was reintroduced here.

Experiment - The material in aqueous solution after chloroform treatment was precipitated by barium acetate; the precipitate was centrifuged, taken up in butyl alcohol and used for the acid and potassium hydroxide treatment (penultimate stage in Table 3).

Results - This barium treatment did not appear to improve the product.

Batch M5.

Idea.- The use of the quinidine salt to characterise oestrone sulphate (Butenandt and Hofstetter, 1939) suggested that quinidine salts might be useful for the separation of other conjugates.

Experiment - The total neutral fraction was treated in aqueous solution with quinidine hydrochloride and the precipitated quinidine salts were put/

put through a charcoal chromatogram.

Result - No separation was achieved. Further work is, however, desirable.

Batches M8-M11.

Chance Discovery - When the total neutral fraction of batch M8 was taken up in hot methyl alcohol to transfer it to another flask and the solution cooled, a partly crystalline precipitate formed. It was hoped that some useful separation might have been achieved.

Experiment - The above mentioned precipitate was filtered off and recrystallised from methyl alcohol. The product was water-soluble and contained organic sulphate and oestrogen (Kober test). Subsequent work[x] showed that this probably contained much potassium p-tolyl sulphate. Batches M9-M11 were worked up similarly. Both methyl alcohol-insoluble and methyl alcohol-soluble fractions were put through the remaining stages of the original method. In no case was crystalline material obtained.

Result - Separation of a 'methyl alcohol-insoluble' fraction is apparently of no value.

Batch /

x see p. 116.

Batch M11 (Later Stages)

Idea - To improve Schachter's purification via the barium salts. Schachter's transformation of the water-insoluble barium salts to water-soluble potassium salts involved dissolving the barium salts in butyl alcohol and shaking with aqueous acid.

$$Ba(XSO_4)_2 + HCl \rightarrow HXSO_4 + BaCl_2$$

in BuOH in water in BuOH in water

The barium was removed in the aqueous layer and the free acid sulphates $HXSO_4$ were transformed into potassium salts by shaking with potassium hydroxide solution.

$$HXSO_4 + KOH \longrightarrow KXSO_4 + HOH$$

in BuOH in water in BuOH

The following single stage procedure should save time and avoid the danger of hydrolysis of the sulphates at the acidification stage.

Experiment - The barium salts were dissolved in butyl alcohol and the solution shaken with aqueous potassium sulphate solution.

$$Ba(XSO_4)_2 + K_2SO_4 \longrightarrow KXSO_4 + BaSO_4$$

in BuOH in water in BuOH solid

The butyl alcohol solution was separated by centrifuging from the aqueous layer and the precipitated barium sulphate, washed with water and evaporated.

The/

The product on crystallisation from aqueous acetone gave a crisp white solid, m.p. 217-218°(dec.) which may have been crude $KZSO_4$.

Result - Promising. This method appears satisfactory for the transformation of barium to potassium salts, and may be useful if it is found that the precipitation of barium salts is of value in separating different groups of conjugates.

Batch M12 (first modification)

Idea - Since the sulphates were ultimately to be isolated as potassium salts, potassium should be introduced as soon as possible in the separations.

Experiment - Potassium hydroxide was used instead of sodium hydroxide in all the operations outlined in Table 2. No difficulties were encountered.

Result - This modification was satisfactory and was incorporated in the standard method.

Batch M12 (second modification)

The Water-Separation.

Chance Discovery - The total neutral fraction from this batch (53 l., collected 3 weeks before foaling) was left to stand for some days in alkaline aqueous solution in the refrigerator. A white solid separated.

This/

This fortunate accident suggested the separation of the crude material into 'water-insoluble' and 'water-soluble' fractions which was done with most subsequent batches.

Experiment - The solid ('water-insoluble' fraction) was separated by centrifuging and washed with ice-water. After drying it was a light brown powder (weight 8.35 g.). Attempts to crystallise this solid from water or ethyl alcohol, or to form a p-toluidine salt directly were unsuccessful. Extraction with 98% acetone (see Part 3, section 1) yielded much crude potassium Z sulphate.

The supernatant aqueous solution ('water-soluble fraction') was put through the remainder of Schachter's treatment - product, 8.0 g. light brown solid.

Result - This separation of 'water-insoluble' and 'water-soluble' fractions was obviously valuable and was incorporated in the standard method of working up. In subsequent batches the total neutral fraction was dissolved in a minimum of boiling water, the solution was cooled to 0° and the two fractions were then separated by centrifuging.

(The term 'water-insoluble fraction' although convenient is a misnomer; the fraction should really be/

be called 'the fraction sparingly soluble in water at 0°').

Batch N1.

Idea - It was thought that time and labour might be saved if the alkali washings of the original butyl alcohol extract were omitted, and all acidic material was removed by exhaustive ether washing of the acidified aqueous solution of the crude residue from the butyl alcohol extracts.

Experiment - The urine was extracted with butyl alcohol in the usual way, the extracts washed once with water and evaporated to dryness. The residue was taken up in water, extracted twice with ether, acidified as usual to pH 1.5 and then extracted eight times with ether. The aqueous solution was basified and extracted with butyl alcohol as usual.

Result - The product so obtained was more sticky than usual, and at first sight the modification seems unsatisfactory. Controls would, however, be necessary to give information for a definite judgment.

Batch N1 (second modification)

The Potassium Acetate Separation

Idea - It was thought that the use of potassium hydroxide instead of sodium hydroxide for washings (as described for Batch M12) was insufficient to ensure that/

that potassium was the only cation present in the desired conjugates.

The 'water-insoluble' fraction was therefore dissolved in water and salted out by the addition of an equal volume of saturated aqueous potassium acetate solution.

Experiment - The water-insoluble fraction was dissolved in water (15 ml.) at 100° and saturated aqueous potassium acetate solution (15 ml.) was then added. A dirty white precipitate separated and increased in bulk on cooling to room temperature. The precipitate was centrifuged and washed twice with its own volume of half-saturated potassium acetate solution. The washed precipitate was dissolved in butyl alcohol saturated with water (50 ml.) and this solution washed with water (3 x 5 ml.) to remove potassium acetate, and evaporated to dryness. The product (345 mg. nearly white powder) was used for the 98% acetone crystallisation (Part 3, section 1).

Result - This operation was satisfactory, and with subsequent batches was used before the water separation (see Batch N11).

Batch N11 /

Batch N11.

Idea - When the solution of the crude neutral fraction in a minimum of boiling water was cooled, only a very small gelatinous precipitate formed. It was thought that more satisfactory results might be obtained if the precipitation by half saturation with potassium acetate was introduced before the water-separation.

Experiment and Result - This was done and the product was satisfactory. Schachter's method with the successful modifications to date constitutes our 'Standard Method' which is detailed in Part 2, section 7.

Batches N24, N36, 01, 04, 06, 09-012, B13.

The Standard Method was used for these batches.

Batch 07.

Idea - It had been found that the p-toluidine salts of simple phenolic sulphates (e.g. phenyl sulphates) were insoluble in chloroform, whereas p-toluidine Z sulphate was known to be freely soluble in this solvent.

It was proposed to treat the neutral fraction in aqueous solution with p-toluidine hydrochloride and extract with chloroform in the hope that this would remove some or all of the steroid sulphates while/

while leaving the phenolic sulphates of lower molecular weight in aqueous solution.

Experiment - An aqueous solution of the neutral fraction was divided into two equal parts,(each assumed to contain 3.1 g. conjugate). One was extracted with butanol in the usual way as a control; the other was brought to pH 7 and p-toluidine hydrochloride (3.1 g.) in water (20 ml.) added. The cloudy solution was extracted with chloroform (3 x 100 ml.) and the chloroform back washed with water (1 x 30 ml.) and evaporated to dryness. The chloroform soluble p-toluidine salts so obtained formed a brown gum (1.05 g.). This fraction gave a strong Kober reaction; on hydrolysis with acid it gave only a faint cresol-like smell. Attempts to crystallise this fraction from chloroform, chloroform-ether and ethyl alcohol were all unsuccessful.

The aqueous solution from the chloroform extractions was heated to remove dissolved chloroform, cooled and extracted with butyl alcohol (4 x 80 ml.). The butyl alcohol solution was washed with water (2 x 30 ml.) and evaporated to dryness, giving a pale brown solid (0.64 g.). This fraction gave a faint Kober reaction, and on heating with acid a strong cresol-like smell. The arrival of the very large batch of urine from Messrs British Drug Houses Ltd. prevented/

prevented further work on these fractions.

Results - This method appears promising as a possible means of separating steroid sulphates (including oestrone sulphate) from non-steroid sulphates. The subsequent discovery that p-toluidine Y sulphate is insoluble in chloroform shows that all steroid sulphates will not be found in the chloroform-soluble fraction.

2, 7. STANDARD METHOD FOR WORKING UP URINE.

This method has been developed from that of Schachter, by a somewhat random process of trial and error, as described in the preceding section. No guarantee can be given that it even approaches an ideal method.

The procedure consists of four stages, viz.:-

A. Extraction of the urine with butyl alcohol.

B. Removal of acidic material from the butyl alcohol extracts.

C. Precipitation of the conjugates from aqueous solution by half-saturation with potassium acetate.

D. Separation of a fraction sparingly soluble in cold water.

The process is outlined in flow sheet form in Tables 4 and 5 (pp.39, 42). Roughly quantitative data on a number of batches of urine are collected in Part 5, section 1.

A. /

A. Butyl Alcohol Extractions

The urine (50 l.) was filtered through glass-wool to remove as much suspended solid as possible, since this tended to cause emulsions during the extractions. It was then extracted with butyl alcohol four times (12.5 l. each time).

Any emulsions which formed could usually be broken by (a) allowing to stand with occasional gentle agitation, (b) adding ethyl alcohol (1-5% of the volume of urine) and allowing to stand with occasional gentle agitation, or (c) centrifuging.

In most cases the four butyl alcohol extracts were combined for stage B, but in two cases they were worked up separately to find out what proportion of material was being removed at each extraction (see Part 5, section 1).

B. Removal of Acidic Material.

(i) The butyl alcohol extracts from stage A (50 l.) were extracted four times with 1 N aqueous potassium hydroxide solution (12.5 l. each time). The first extraction often gave much persistent emulsion; subsequent emulsions did not. The emulsions could usually be broken by (a) allowing to stand, (b) adding saturated potassium acetate solution (10% of the volume of the potassium hydroxide solution) or (c) centrifuging.

The/

The alkali washings were usually discarded, but in a few batches their contents were briefly examined (see Part 5, section 1).

(ii) The alkali-washed butyl alcohol extracts were then washed with water four times (3.0 l. each time). Perfect and rapid separation of butyl alcohol and water layers at this stage could be achieved by adding to the water one-tenth of its volume of saturated potassium acetate solution.

The water washings were discarded and the butyl alcohol solutions were evaporated to dryness in vacuo at temperatures not exceeding 100°.

(iii) The residue from the butyl alcohol was dissolved in water (1500 ml.), extracted four times with ether (1 x 300, 3 x 150 ml.) and cooled to 0°. The solution was then acidified to pH 2 with concentrated hydrochloric acid and rapidly extracted with ether (2 x 250 ml., 2 x 125 ml. previously cooled to 0°),[x] to remove/

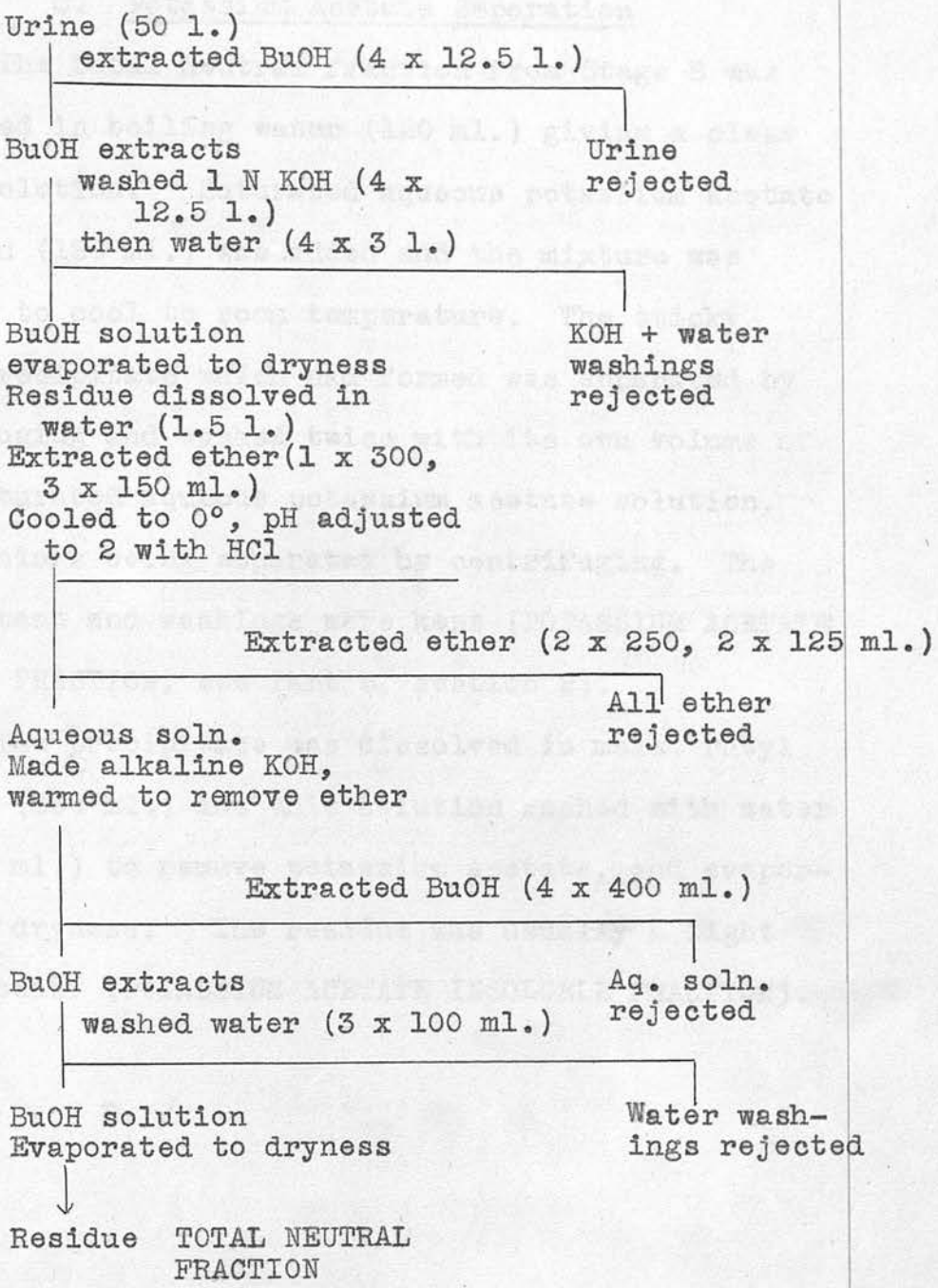
x If the fourth ether washing was coloured, further ether extractions were carried out until the washings were colourless.

remove acidic materials not extracted from butyl alcohol by 1 N aqueous potassium hydroxide. All ether washings were discarded.

(iv) The aqueous solution was basified to litmus with concentrated aqueous potassium hydroxide solution, warmed to evaporate most of the dissolved ether, and cooled to room temperature. It was then extracted with butyl alcohol (4 x 400 ml.) and the butyl alcohol extracts were washed with water (3 x 100 ml.) and evaporated to dryness in vacuo. The residue was freed from traces of butyl alcohol by repeated addition of small quantities of methyl alcohol and evaporation of these in vacuo. The residue usually formed a pale brown friable powder, and was called the TOTAL NEUTRAL FRACTION.

Table 4.

Standard Extraction Method (Stages A and B)



C. Potassium Acetate Separation

(i) The total neutral fraction from Stage B was dissolved in boiling water (120 ml.) giving a clear brown solution. Saturated aqueous potassium acetate solution (120 ml.) was added and the mixture was allowed to cool to room temperature. The sticky brown precipitate which had formed was separated by centrifuging and washed twice with its own volume of half-saturated aqueous potassium acetate solution, the washings being separated by centrifuging. The supernatant and washings were kept (POTASSIUM ACETATE SOLUBLE FRACTION, see Part 5, section 2).

(ii) The precipitate was dissolved in moist butyl alcohol (350 ml.) and this solution washed with water (3 x 50 ml.) to remove potassium acetate, and evaporated to dryness. The residue was usually a light brown powder (POTASSIUM ACETATE INSOLUBLE FRACTION).

D. /

D. Water Separation.

The potassium acetate insoluble fraction from Stage C was dissolved in the minimum of boiling water, the solution transferred to a centrifuge tube, cooled slowly to 0° [x] and the precipitate separated by centrifuging. The precipitate was washed once with a small volume of ice-water by centrifuging, then dissolved in butyl alcohol and the solution evaporated to dryness[xx], giving a pale brown powder referred to as the 'WATER INSOLUBLE FRACTION'.

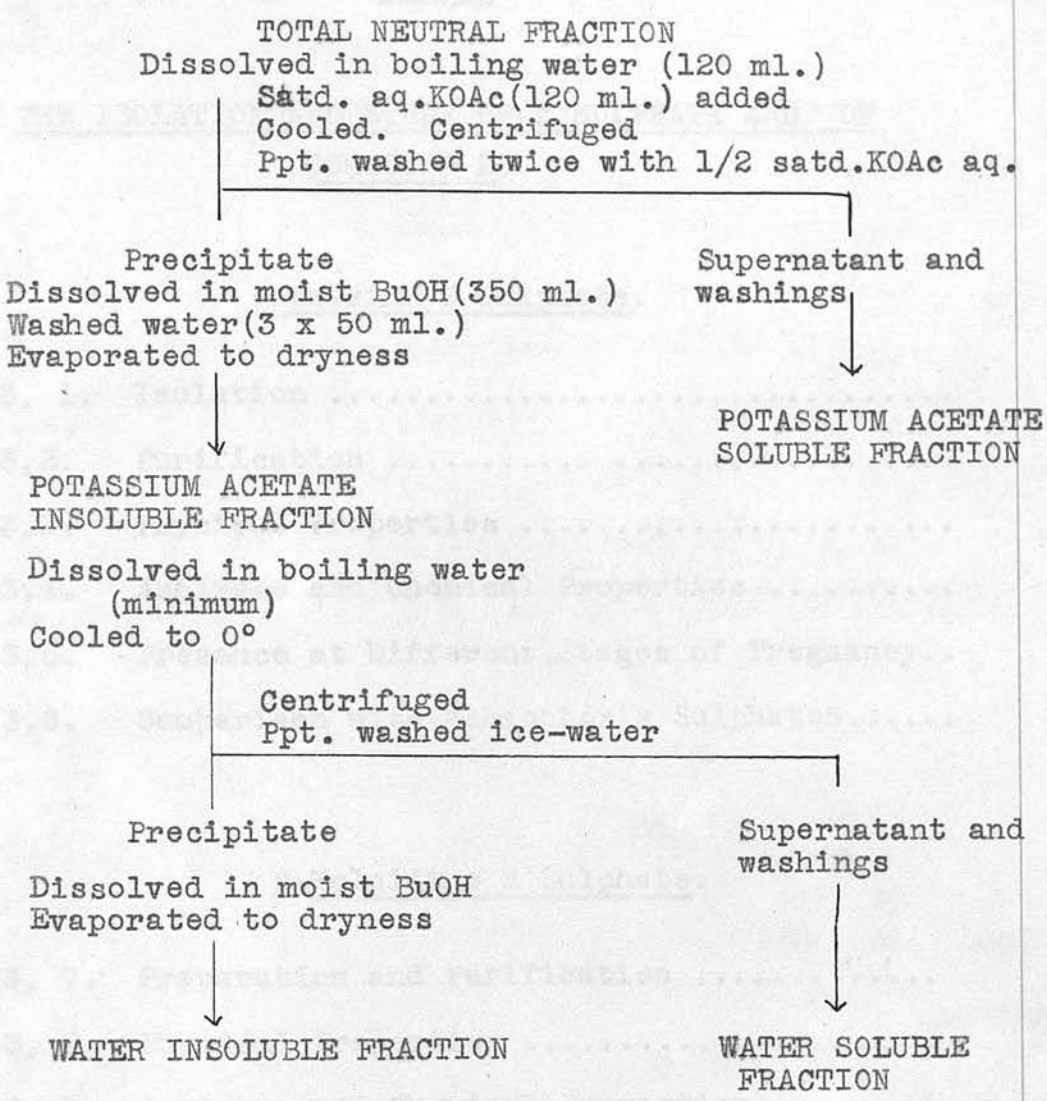
The supernatant and washing were extracted four times with an equal volume of butyl alcohol and the butyl alcohol extracts evaporated to dryness giving the so-called 'WATER-INSOLUBLE FRACTION'.

x In some cases the solution and precipitate were frozen at -10°, and then centrifuged until all the ice just remelted.

xx This was much quicker than drying the pasty precipitate in a desiccator.

Table 5.

Standard Extraction Method (Stages C and D)



PART 3.

THE ISOLATION AND STUDY OF Z SULPHATE AND OF COMPOUND Z.

Potassium Z Sulphate.

p-Toluidine Z Sulphate.

Compound Z /

Compound Z.

3, 1. ISOLATION OF POTASSIUM Z SULPHATE.

Potassium Z sulphate was contained in the 'water-insoluble fraction (see Part 2, section 7). It was obtained from this by a method used by Schachter, viz. repeated extraction with acetone containing 2% v/v of water ('98% acetone') and partial evaporation of the extracts. The method is unsatisfactory, since it is impossible to know what proportions of acetone and water are present at each stage of the evaporation. When supplies of pure potassium Z sulphate become available, a study of the solubility of this compound in acetone:water mixtures of various compositions may enable us to improve the method.

The only good yields of potassium Z sulphate have been obtained from urine obtained within the last three weeks preceding foaling (batches M12 and N36). Melting-points of the fractions from these batches suggest that some substance other than potassium Z sulphate is extracted by the early 98% acetone fractions, while the later 98% acetone fractions give fairly pure potassium Z sulphate (see Table 6). Practical details varied somewhat with different batches.

Experimental /

Experimental Details for a Satisfactory Batch.

The following details are for batch N36 (53 l. of urine collected 0-3 weeks before foaling); volumes and weights are given in Table 6.

The 'water-insoluble' fraction (4.7 g. yellowish solid) was heated under reflux on the boiling water-bath with acetone for 20 minutes and filtered hot. The undissolved solid was used for the next extraction. The filtrate was concentrated by heating on the water-bath under a gentle stream of air until solid began to separate. It was then cooled to 0° in the refrigerator, and the solid filtered, washed with acetone and dried.

Table 6. /

Table 6.

98% Acetone Extraction of Batch N36.

Fraction	Volume of 98% acetone ml.	Volume after concn.	Appearance, wt. and m.p.(a) of solid
1	200	50(b)	Yellowish-white crisp solid. 144 mg., m.p.218-219
2	200	40(b)	White crisp solid. 138 mg. m.p. 220-223.
3	100	10	(This and all subsequent fractions) white crystals. 64 mg., m.p. 231-232.
4	100	50	60 mg., m.p. 229-230.
5	100	100(c)	76 mg., m.p. 227-229.
6	100	10	83 mg., m.p. 237.
7	100	3	29 mg., m.p. 238-240.
8	100	5	38 mg., m.p. 234-235.

(a) Corrected. All samples decomposed on melting and sintered some degrees below the m.p.

(b) These filtrates were pale yellow; all others were colourless.

(c) No concentration solid separated immediately after hot filtration.

Other Batches.

Results for other batches are detailed in Table 7. In some cases where no solid separated on concentration of the aqueous acetone solution material could be precipitated by cautiously adding more acetone; such precipitates were usually of low melting- point. In some cases attempts were made to get more material by further concentration of the aqueous acetone after the first crop of sulphate had been filtered. Second crops so obtained were usually so impure as to be worthless.

There was usually a considerable residue which did not dissolve in 98% acetone even after repeated extraction.

Table 7 /

Table 7.

98% Acetone Extractions - Other Batches.

Batch	Weeks before foaling	Vol. of urine (l.)	Wt.of water-insol. fraction (g.)	Extracts (ml.98% acetone)	Products (b)
M1-2	16	27.4	(a)	1 x 100	48 mg.,m.p.204-207. Water crystallisation gave material, m.p. 230-231, which was probably $KZSO_4$
M3	15	11.6	(a)	1 x 125	Yellowish cryst. solid, m.p.190-193.
M4	15	6.6	(a)	1 x 20	Amorphous solid m.p. 201-203.
M12	3-4	53.1	8.0	5 x 400	1400 mg.,white crisp,amorphous solid, m.ps. 213-223.
N1	13	6.6	0.35	4 x 15	nil
N5	12	8.0	0.90	4 x 50	100 mg. white crisp solid, m.p. 210-215.
N11	11	11.2	3.6	1 x 180 5 x 100	173 mg., m.p. 212-215.
N24/					

(a) Water separation not carried out on these batches; 98% acetone treatment done on material put through treatment of Table 3 (p.25)

(b) M.p's uncorrected for M batches; corrected for all others.

Table 7 (contd.)

Batch	Weeks before foaling	Vol. of urine (l.)	Wt. of water-insol. fraction (g.)	Extracts (ml. 98% acetone)	Products
N24	8-9	14.7	2.1	4 x 100	142 mg. pinkish white solid, m.p. 212-222
01-3	11-14	52.0	2.6	3 x 50	43 mg., m.p. 201-205.
06	8	18.0	1.55	1 x 30	Nearly all material dissolved in 98% acetone. No satisfactory product obtained
09	2	17.5	1.6	1 x 50	
B13	(few)	200	19.4	See Part 4, section 1.	

The great variation in the nature of the extracts, even from animals at about the same stage of pregnancy is well illustrated by Tables 6 and 7. Compare the results with batches M12 and N36 (which gave potassium Z sulphate), and batch 09 (which gave none).

3, 2. PURIFICATION OF POTASSIUM Z SULPHATE.

Potassium Z sulphate obtained by the 98% acetone treatment could be crystallised from water or from ethyl alcohol and is apparently dimorphous. Crystallisation from ethyl alcohol always gives Form A of m.p. 244-245° (dec., corr.); crystallisation from water gives sometimes Form A but more often Form B of m.p. 216-217° (dec., corr.). Each form appears to contain one molecule of water of crystallisation (see p. 58)

Since p-toluidine Z sulphate shows no such complications, it was chiefly used for the purification and characterisation of Z sulphate (see section 7). Pure potassium Z sulphate can be obtained if necessary by treatment of the pure p-toluidine salt with potassium hydroxide (see section 9).

Attempts to purify material of poor quality.

Many attempts were made to get more pure potassium Z sulphate from impure material obtained in the 98% acetone treatment, and from the mother liquors of water or alcohol crystallisations of purer fractions. The attempts included:

(a) recrystallisation of potassium salts from water or ethyl alcohol.

(b) transformation of potassium salts into p-toluidine/

toluidine salts and reconversion of these into potassium salts.

(c) recrystallisation of p-toluidine salts from water.

Most of these attempts were failures, repeated attempts at crystallisation giving gels or solids of low melting-point. One of the few fairly successful attempts is described below (Experiment 104).

These failures suggest that potassium Z sulphate is accompanied by several other substances of very similar properties and that it decomposes on repeated heating with solvents.

It should be noted particularly that potassium Z sulphate of low quality (m.p. less than 210°) gave with p-toluidine hydrochloride in aqueous solution either amorphous precipitates or gels. It would appear that some of the other sulphates present resemble Z sulphate in the form of their p-toluidine salts as well as their potassium salts.

Experiment 104 - Crude material from a second crop in the 98% acetone extraction (268 mg., m.p. 207-208) gave a gel when its hot aqueous solution was cooled. The solution was reheated, and an excess of p-toluidine hydrochloride added. On cooling the mixture gelled again. On long standing the material became semi/

semi-solid and could be separated from the supernatant by centrifuging. The solid so obtained (presumably p-toluidine salts) was treated with potassium hydroxide in aqueous alcoholic solution, and on partial evaporation the solution deposited 30 mg. of fairly pure potassium Z sulphate (m.p. 231-234° dec., corr.). One subsequent experiment gave similar results.

3, 3. PHYSICAL PROPERTIES OF POTASSIUM Z SULPHATE.

Melting Points (corrected) [x]

Form A- 244-245° (browning at 234° and sintering at 241°)

Form B- 216-217° (browning at 205°)

The melting-point of Form A fell by 15-20° on keeping in a vacuum desiccator for 4 months.

Crystalline Form.

Form A- Rosettes of feathery needles (from ethyl alcohol)

Form B- Clusters of very small prisms (from water)

x Unless otherwise stated melting-points were taken in a conventional liquid bath apparatus stirred by convection currents. Rate of heating, 3-6° per min. The melting-points of many organic sulphates are dependent on the rate of heating.

Solubilities/

Solubilities. Very approximate

	At b.p.of solvent. mg./ml.	At 0° mg./ml.
Water	10-11	3
Ethyl alcohol	9	2.5
Butyl alcohol	10	7
Chloroform	0.03	0.03

Salting-out.

This experiment was carried out to see how completely the potassium acetate treatment (Part 2, section 7) precipitated potassium Z sulphate.

Potassium Z sulphate (9.6 mg.) was dissolved in water (5.0 ml.) and saturated aqueous potassium acetate solution (5.0 ml.) was added. The precipitated potassium Z sulphate weighed 7.5 mg., i.e. 79% of the original quantity (m.p. 232°, dec., corr.).

Dimorphism.

A few preliminary experiments were carried out on the nature of the two forms of potassium Z sulphate, using material which had been purified via the p-toluidine salt. The matter was not investigated thoroughly since the p-toluidine salt was so satisfactory for purification and identification.

The following facts are certain.

(1) /

(1) Analytical figures on both forms agree fairly well with those required by $C_{21}H_{31}O.SO_4K,H_2O$ (see next section).

(2) The melting point of each form is unchanged after heating to 80° in vacuo for 1 hour over P_2O_5

(3) Both forms on treatment with p-toluidine hydrochloride give the same p-toluidine salt (m.p. 195-197°, corr.), which is identical (mixed m.p.) with that from which the potassium salt was obtained.

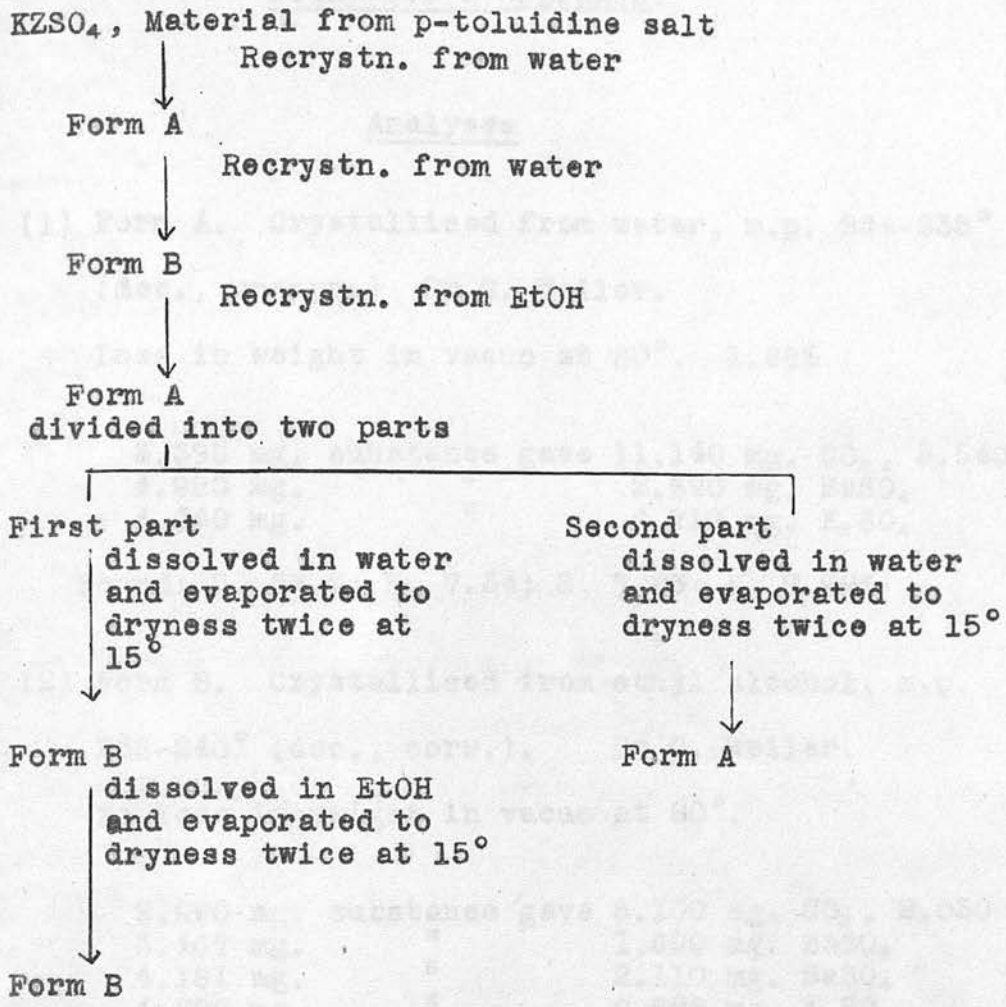
It is very probable, therefore, that the two forms are merely polymorphic modifications of the same substance; they are not different hydrates and it is unlikely that they are formed from one another by intramolecular rearrangement.

Interconversion of Two Forms

The two forms are certainly interconvertible, but the conditions in which they are transformed into one another are not certain.

A series of experiments may be summed up as follows:

$KZSO_4$ /



The reason for the inconsistent behaviour of the two forms is as yet unknown.

3, 4 /

3, 4. ANALYSES AND CHEMICAL PROPERTIES OF POTASSIUM Z SULPHATE.

Analyses

(1) Form A. Crystallised from water, m.p. 234-235° (dec., uncorr.) Dr G. Weiler.

Loss in weight in vacuo at 80°, 1.68%

5.399 mg. substance gave 11.140 mg. CO_2, 3.540 mg. H_2O
4.980 mg. " 2.620 mg. $BaSO_4$
4.040 mg. " 0.719 mg. K_2SO_4

Found: C, 56.3; H, 7.34; S, 7.23; K, 7.99%

(2) Form B. Crystallised from ethyl alcohol, m.p. 238-240° (dec., corr.). Dr G. Weiler.

No loss in weight in vacuo at 80°.

2.990 mg. substance gave 6.100 mg. CO_2, 2.030 mg. H_2O
3.467 mg. " 1.690 mg. $BaSO_4$
4.181 mg. " 2.110 mg. $BaSO_4$
4.290 mg. " 0.828 mg. K_2SO_4

Found: C, 55.7; H, 7.60; S, 6.69, 6.93; K, 8.66%.

(3) Form B. Purified via p-toluidine salt, and crystallised from ethyl alcohol. M.p. 244-245° (dec., corr.). Mrs G.F. Marrian.

(C, H and K determined on same sample)

2.246 mg. substance gave 4.592 mg. CO_2, 1.499 mg. H_2O, 0.442 mg. K_2SO_4
2.341 mg. substance gave 4.787 mg. CO_2, 1.565 mg. H_2O, 0.442 mg. K_2SO_4.

Found/

Found: C, 55.8, 55.8; H, 7.47, 7.48; K, 8.83, 8.47%

Calculated	C	H	S	K
$C_{21}H_{31}O.SO_4K + H_2O$	55.7	7.35	7.08	8.64%
$C_{21}H_{33}O.SO_4K + H_2O$	55.5	7.76	7.05	8.60%
$C_{21}H_{31}O.SO_4K$	58.0	7.19	7.37	9.00%
$C_{21}H_{33}O.SO_4K$	57.8	7.62	7.34	8.95%

Since analyses of p-toluidine Z sulphate and of Compound Z itself indicate that Z contains only two oxygen atoms, it must be assumed that potassium Z sulphate contains one molecule of water of crystallisation which is not removed by crystallisation from ethyl alcohol or by heating at 80° in vacuo over P_2O_5.

Reactions.

Sodium Fusion Test for Elements.

This test showed that potassium Z sulphate contains sulphur but not nitrogen or halogens.

Formation of Inorganic Sulphate by Acid Hydrolysis.

Potassium Z sulphate on boiling with hydrochloric acid, cooling and adding barium chloride, gave a white precipitate, which did not dissolve on heating and was presumably barium sulphate.

Presence/

Presence of Chromogenic Oestrogen

Potassium Z sulphate gave no colour in the phenol-sulphuric acid oestrogen test of Kober (1931) as modified by Cohen and Marrian (1934).

Potassium Z sulphate (0.2 mg.) and Kober's phenol-sulphuric acid reagent (0.5 ml.) were heated in the boiling water-bath for 10 minutes, chilled in ice, and diluted with water (2.0 ml.). The mixture was heated on the boiling water-bath again for 2 minutes and chilled. The resulting solution was colourless.

The following chemical properties of potassium Z sulphate are dealt with in later sections of this Part.

Formation of p-toluidine Z sulphate - Section 7.
Formation of salts with other cations - Section 10.
Hydrolysis - Section 11.

3, 5. PRESENCE OF POTASSIUM Z SULPHATES AT DIFFERENT STAGES OF PREGNANCY.

The only batches of mare's pregnancy urine which gave large quantities of potassium Z sulphate were two (M12 and N36) collected 3 weeks or less before foaling; these yielded 27 mg./l. and 28 mg./l. crude potassium Z sulphate respectively.

Other batches collected from 4-16 weeks before foaling have given smaller quantities of cruder

material/

material from which it was more difficult to obtain potassium Z sulphate. This suggests that the amount of potassium Z sulphate excreted, and the ratio of Z sulphate to total sulphate both increase in the last few weeks before foaling.

3,6. COMPARISON OF POTASSIUM Z SULPHATE WITH SCHACHTER'S SULPHATES.

Direct comparison of potassium Z sulphate with Schachter's sulphates was impossible since no samples of the latter were available. The melting-points of the two forms of potassium Z sulphate and of Schachter's sulphates are tabulated below, but no conclusions can be drawn as to possible identities.

			M.p.
$KZSO_4$	Form A	Klyne	244-245°(dec.,corr.)
"	Form B	"	216-217° "
$KASO_4$		Schachter	215.5-216.5°
$KBSO_4$		"	224-225°
$KCSO_4$		"	240-250°

Comparison of the hydrolysates A and Z (section 15) suggests that these two compounds are probably <u>not</u> identical.

3, 7 /

3, 7. PREPARATION AND PURIFICATION OF p-TOLUIDINE Z SULPHATE.

The use of p-toluidine salts for the characterisation of mono-alkyl sulphates was suggested by Barton and Young (1939). The p-toluidine salt has been found most convenient for the characterisation and purification of Z sulphate, since it is sparingly soluble in water and is readily recrystallised from this solvent.

Preparation.

$$(C_{21}H_{31}O.SO_4)^-K^+ + (CH_3.C_6H_4.NH_3^+)Cl^- =$$

$$(C_{21}H_{31}O.SO_4)^- (CH_3.C_6H_4.NH_3)^+ + K^+Cl^-$$

Potassium Z sulphate (301 mg., 0.60 millimols; m.p. 238-239°, dec., corr.) was dissolved in boiling water (50 ml.) and a solution of p-toluidine hydrochloride (185 mg., 1.3 millimols) in water (5 ml.) was added. A copious precipitate formed. Water was then added at 100° until the precipitate just dissolved (total volume 90 ml.) and the solution was cooled to 0°. The micro-crystalline precipitate which separated was filtered and dried - weight 293 mg. 87% of theory; m.p. 190-192° (corr.).

Purification /

Purification.

p-Toluidine Z sulphate obtained from potassium salt of good quality was easily purified by re-crystallisation from water. Samples of p-toluidine salt obtained from potassium salt of poor quality could not be purified thus.

3, 8. PHYSICAL PROPERTIES OF p-TOLUIDINE Z SULPHATE.

Melting-Point (corrected) 195-197° (softening at 186°)

Crystalline Form

Fine white needles (from water)

Specific Rotation

$[\alpha]_D^{19}$ + 32° ± 1° [x] (C = 3 in chloroform)

Solubilities

	Very approximate	
	At boiling point of solvent mg./ml.	At room temperature (15-20°) mg./ml.
Water	3.5	0.5
Chloroform		∠ 30

x All specific rotations are given as mean ± twice standard error.

3, 9. ANALYSES AND CHEMICAL PROPERTIES OF p-TOLUIDINE Z SULPHATE

Analyses

Early analyses by Dr G. Weiler gave discordant results; they showed, however, that p-toluidine Z sulphate did not lose weight on heating in vacuo at 80°.

Analyses by Mrs G.F.Marrian

2.881 mg. substance gave 7.099 mg. CO_2, 2.093 mg. H_2O
3.176 mg. " " 7.816 mg. CO_2, 2.300 mg. H_2O
5.900 mg. " " 0.151 ml.(corr.) N_2 at 21°,752 mm.
5.586 mg. " " 0.137 ml.(corr.) N_2 at 22°,748 mm.
2.901 mg. " " 1.341 mg. $BaSO_4$
3.184 mg. " " 1.484 mg. $BaSO_4$

Found:	C	H	N	S
	67.2	8.13	2.76	6.35%
	67.2	8.10	2.91	6.40%

Calculated:		C	H	N	S
$C_{21}H_{31}O.SO_4^-, CH_3.C_6H_4.NH_3^+$		66.8	8.20	2.78	6.36%
$C_{21}H_{33}O.SO_4^-$,	"	66.5	8.57	2.77	6.34 %
$C_{21}H_{33}O_2.SO_4^-$,	"	64.5	8.31	2.69	6.14%
$C_{21}H_{35}O_2.SO_4^-$,	"	64.2	8.66	2.68	6.12%

Reactions

Hydrolysis

The hydrolysis of p-toluidine Z sulphate to compound Z is described in section 3, 11.

Transformation/

Transformation of p-Toluidine Sulphate into Potassium Z Sulphate

This reaction was carried out by treating the p-toluidine salt with excess potassium hydroxide in aqueous solution and removing the free base, p-toluidine, by extraction with ether.

$$(CH_3.C_6H_4.NH_3)^+ (ZSO_4)^- + K^+OH^- = K^+(ZSO_4)^- + CH_3.C_6H_4.NH_2 + HOH$$

p-Toluidine Z sulphate (100 mg.) in warm water (100 ml.) was treated with potassium hydroxide (1.0 g.) in water (5 ml.), and p-toluidine was extracted with ether (4 x 25 ml.). The aqueous solution was heated to drive off dissolved ether, cooled, and extracted with butyl alcohol (2 x 50 ml., 2 x 25 ml.). The butyl alcohol extracts on evaporation gave crude potassium Z sulphate (86 mg. 90% of theory; m.p. 222-224° (dec., corr.).

Since p-toluidine Z sulphate can easily be purified, this method provides the best means of getting pure potassium Z sulphate.

3,10. OTHER SALTS OF Z SULPHATE

Barium Z Sulphate.

This was obtained as a white solid by mixing hot aqueous solutions of potassium Z sulphate and barium chloride and cooling. On recrystallisation from water it was a white micro-crystalline solid (leaflets?); m.p./

m.p. 142-144°(dec.). Solubilities in water 1 mg./ml. at 100°, 0.6-0.7 mg./ml. at 0°.

Quinidine Z Sulphate.

This was obtained as a white precipitate on mixing aqueous solutions of potassium Z sulphate and quinidine hydrochloride at room temperature. On heating with water it gave a sticky liquid which resolidified on cooling (m.p. 163-165° after slow heating). Attempts were made to crystallise this salt from other solvents but it proved intractable and was not further investigated. Contrast quinidine oestrone sulphate (Butenandt and Hofstetter, 1939).

3, 11. PREPARATION AND PURIFICATION OF COMPOUND Z.

Preparation

Compound Z was prepared by acid hydrolysis of its sulphate (potassium or p-toluidine salt).

$$C_{21}H_{31}O.SO_4^-B^+ + H_2O = C_{21}H_{32}O_2 + B^+ + H^+ + SO_4^{--}$$

Best Method.

The most satisfactory method was as follows:-

p-Toluidine Z sulphate (154 mg.) was dissolved in hot water (100 ml.), hydrochloric acid (2M, 100 ml.) was added, and the mixture was heated in the boiling water-bath. The initially clear solution became cloudy/

cloudy after 10 minutes' heating, and a precipitate formed, which gradually increased in bulk. After 2 hours' heating the mixture was cooled and extracted with ether (1 x 100 ml. and 3 x 50 ml.). The ether extracts were washed with hydrochloric acid (1M, 2 x 30 ml. to remove p-toluidine), sodium bicarbonate (0.5 M, 30 ml.; to remove acid) and water (3 x 30 ml.). The extracts on evaporation to dryness yielded crude Compound Z, 88 mg. (91% of theory), m.p. (corr.) 197-202° after sintering at 184°.

Comparison of all methods used.

This method is compared with other less satisfactory ones, in Table 8.

Table 8 /

Table 8.

Methods for Hydrolysis of Z Sulphates

Method	Salt used	Acid reagent used	Time of heating at 100° (mins.)	Yield and m.p. of Z produced
1	K	1M. aq. HCl	50	49%; m.p. 188-193° (uncorr.)
2	K	0.33 ml. HOAc, 100 ml H_2O 15 g. $BaCl_2$ (pH 2.65) (a)	240	Solid did not dissolve. Product shown to be $Ba(ZSO_4)_2$
3	K	10 ml. EtOH + 5 ml. 2.5 M aq. H_2SO_4 (b)	60	66%; m.p. 184-187° (uncorr.)
4	K	1 M aq. HCl + CCl_4 (c)	70	68%; m.p. 194-197° (uncorr.)
5	p-toluidine	1 M aq. HCl (d)	120	91%; m.p. 197-202° (corr.)

(a) Following method developed by Talbot, Ryan and Wolfe (1943) for the hydrolysis of sodium dehydroisoandrosterone sulphate. Method useless here.

(b) Following method used by Butenandt and Westphal (1936) for allopregnanolone sulphate.

(c) Simultaneous hydrolysis and extraction (Callow et al., 1939).

(d) Best method; detailed above.

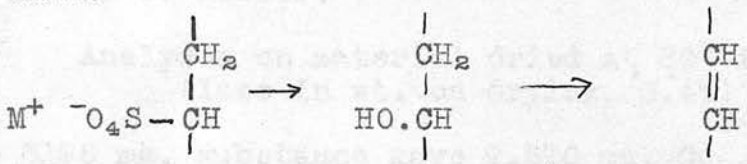
The fact that a high yield of good quality material was obtained from the p-toluidine salt suggests that the nature of the cations present may be/

be of importance in these hydrolyses. It is hoped to investigate this matter further with simpler sulphates.

Purification

Compound Z was usually purified by recrystallisation from aqueous ethyl alcohol (approximately 1:1 by volume).

It was suspected that Z (partial formula II) might be contaminated with the unsaturated compound (III) obtained by hydrolysis and subsequent dehydration thus:-



I. II. III.

In aqueous alcohol the unsaturated compound III would be less soluble than II, but in a non-polar solvent such as benzene, III would be more soluble than II. An attempt was therefore made to crystallise Compound Z from benzene as follows. Compound Z (26 mg., m.p. 192- poor quality material) on recrystallisation from benzene (1 ml.) gave 8 mg. of material (m.p. 203-207°). The yield was so low that no further crystallisations from benzene were attempted.

3, 12 /

3, 12. PHYSICAL PROPERTIES OF COMPOUND Z

Melting-point (corrected) 205-207°

Crystalline Form. Glistening leaflets (from aqueous ethyl alcohol)

Specific Rotation.

$[\alpha]_D^{18}$ + 50° ± 5° (C = 1.4 in ethyl alcohol)

3, 13. ANALYSES AND CHEMICAL PROPERTIES OF COMPOUND Z

Analyses

(1) Dr G. Weiler.

Analyses on material dried at 80° in vacuo. (loss in wt. on drying, 3.4%)

3.196 mg. substance gave 9.310 mg. CO_2, 2.930 mg. H_2O

3.929 mg. " " 11.490 mg. CO_2, 3.550 mg. H_2O

Found: C, 79.5, 79.8; H, 10.26, 10.11%

0.373 mg. substance in 3.513 mg. camphor; D = 12.8°

0.684 mg. " 6.858 mg. " ; D = 11.4°

Found, M.W. 307, 325.

(2) Mrs G.F.Marrian.

Analyses on material dried at 80° in vacuo.

1.902 mg. substance gave 5.610 mg. CO_2, 1.777 mg. H_2O.

2.359 mg. " " 6.901 mg. CO_2, 2.158 mg. H_2O.

Found: C, 80.5, 79.8; H, 10.45, 10.23%.

Calculated/

Calculated.	C	H	M.W.
$C_{21}H_{32}O_2$	79.7	10.19%	316
$C_{21}H_{34}O_2$	79.2	10.76%	318
$C_{21}H_{34}O_3$	75.4	10.25%	334
$C_{21}H_{36}O_3$	75.0	10.79%	336

It is certain from the carbon analyses that Compound Z contains only two atoms of oxygen in its molecule.

Reactions.

Observations only are given in this section. Conclusions are discussed in Section 3, 16.

Precipitation with Digitonin - Z (0.5 mg.) in 90% ethyl alcohol (0.2 ml.) mixed with digitonin (0.2 ml.) of 1% solution in 90% ethyl alcohol) gave a considerable white precipitate within 30 secs.

Colour Tests.

Each test was carried with 0.5 mg. Z, and at room temperature unless otherwise stated.

Concentrated Sulphuric Acid - Z was treated with concentrated sulphuric acid (0.2 ml.); in a few seconds it gave a strong yellow colour with a green fluorescence. The colour became orange-yellow after 5 minutes, and deep orange after 1 hour.

Liebermann/

Liebermann-Burchardt Test - Z was dissolved in chloroform (0.2 ml.) and acetic anhydride (2 drops) and concentrated sulphuric acid (0.2 ml.) were added. On shaking, the lower (acid) layer immediately became deep red, while the upper layer was colourless.

Salkowski Test - Z was dissolved in chloroform (0.2 ml.) and concentrated sulphuric acid (0.2 ml.) was added. The lower (acid) layer became faintly orange; the upper layer was colourless with a yellow green fluorescence.

Tetranitromethane - Z dissolved in tetranitromethane gave no colour.

Zimmermann Ketosteroid Test - Samples of Z in ethyl alcohol (0.1 ml.) with m-dinitrobenzene (0.1 ml.) 2% alcoholic solution) and potassium hydroxide (0.1 ml., 2.5 N alcoholic solution) were kept at 25° for different times and then diluted with ethyl alcohol (5.0 ml.) The colours developed were as follows:-

Time at 25° mins.	Colour with Z	Reagent Blank
20	reddish-purple	pale brown
40	ditto, slightly deeper	ditto
80	ditto, deeper	dull brown

Millon's Test - An aqueous alcoholic solution of Z treated with Millon's reagent gave no colour either in the cold, or on heating to 100° for 50 seconds.

Attempted/

Attempted Preparation of Semicarbazone.

An attempt was made to prepare a semicarbazone from Compound Z by the usual method. Z was recovered unchanged.

Compound Z was treated with semicarbazide hydrochloride (5.6 mg., 2 moles) and hydrated sodium acetate (8.0 mg., 2.2 moles) in water (0.5 ml.) and left at room temperature 72 hours. The product obtained by evaporation and dilution with water was recrystallised twice from aqneous ethyl alcohol; it then had m.p. 199-201° (corr.) and was shown by analysis to contain no nitrogen. The mixed melting-point with Z was 198-200° (corr.) and the material was apparently unchanged Z.

The formation of Z acetate is described in the following section.

3, 14. COMPOUND Z ACETATE

Compound Z on acetylation in the usual manner gave a mono-acetate.

Preparation and Recrystallisation.

Compound Z (7.8 mg.) was heated at 100° for 2 hours with acetic anhydride (1 ml.) and pyridine (1 ml.). Much water was added, and the white flocculent precipitate obtained was filtered, washed and dried. This material was recrystallised twice from/

from aqueous ethyl alcohol (approx. 1:1 by volume).

Purification by Chromatography.

Impure Z acetate was readily purified by chromatography, using the methods elaborated for the acetates of the adrenal steroids by Reichstein and his co-workers (Steiger and Reichstein, 1938a; Reichstein and von Euw, 1938b, 1941; von Euw and Reichstein, 1942).

Miscellaneous crude samples of Z (33 mg.) were treated with acetic anhydride (3.0 ml.) and pyridine (3.0 ml.) at room temperature for 17 hours. The solvents were removed in vacuo and the crude acetate dissolved in ether. The ethereal solution was washed with dilute hydrochloric acid, dilute sodium carbonate and water (three times), dried with sodium sulphate and evaporated to dryness, yielding 40 mg. crude Z acetate.

The aluminium oxide (4 g., Merck, standardised according to Brockmann) was prepared for use by heating at 170-180° for one and a half hours to remove water and cooling in a desiccator over phosphorus pentoxide. It was then made into a thin cream with ~~the~~ 50% pentane: 50% benzene[x] and filled into/

x All percentage figures for mixed solvents for chromatograms are % by volume.

into the chromatogram tube, giving a column 65 mm. high and 10 mm. diameter. The crude Z acetate was dissolved in 50% pentane:50% benzene and run on to this column, which was then eluted continuously as follows. For each fraction 10 ml. solvent were used.

Fraction	Solvent	Product
1-3	50% pentane:50% benzene	Traces oil
4	20% " 80% "	Traces oil
5	20% " 80% "	Trace crystals, m.p. ca 140°
6-13	20% " 80% "	18 mg. crystals, m.p.s.156-167° Good Z acetate
14-16	20% " 80% "	7 mg. crystals, m.p's. 152°-159°.
17-22	benzene	Poor Z acetate
23-25	50% benzene:50% ether	Oil

The good Z acetate from fractions 6-13 was re-crystallised twice from aqueous ethyl alcohol.

Physical Properties.

Melting-point (corrected) 163-165° (softening at 160°)

Crystalline Form. Elongated hexagonal leaflets (from aqueous ethyl alcohol)

Analyses /

Analyses

Material dried 2 hrs. at 80° in vacuo over P_2O_5.

Mrs G.F. Marrian.

1.304 mg. substance gave 3.665 mg. CO_2, 1.108 mg. H_2O

1.139 mg. " " 3.190 mg. CO_2, 1.002 mg. H_2O

	% C	% H
Found:	76.7	9.51
	76.4	9.84
Calculated:		
$C_{23}H_{34}O_3$ pregnenolone acetate	77.1	9.56
$C_{23}H_{36}O_3$ pregnanolone acetate	76.6	10.07
$C_{23}H_{38}O_4$ pregnenediol diacetate	74.6	9.51
$C_{25}H_{40}O_4$ pregnanediol diacetate	74.2	9.96

Reactions

Compound Z acetate (0.2 mg.) used in each case.

Concentrated sulphuric acid. - Z acetate was dissolved in the acid (5 drops). Within 1 min. the solution was deep yellow with a green fluorescence. The colour became deeper on standing. After 24 hours the solution was bright red, without fluorescence.

Liebermann/

Liebermann-Burchardt - Z acetate was dissolved in chloroform (5 drops), and acetic anhydride (2 drops) and concentrated sulphuric acid (5 drops) were added. The acid became deep red, with a green fluorescence; the chloroform was colourless.

Tetranitromethane - Z acetate was dissolved in chloroform (5 drops) and tetranitromethane (5 drops) was added. A very faint yellow colour was produced, similar to that of the reagent blank.

Zimmermann Ketosteroid Test - Z acetate was dissolved in ethyl alcohol (0.1 ml.), mixed with m-dinitrobenzene (0.1 ml. 2% alcoholic solution) and potassium hydroxide (0.1 ml. 2.5 N alcoholic solution), kept at 25° for 40 minutes, and diluted with ethyl alcohol. A purplish-red colour was produced. (Reagent blank, pale brown).

3,15. COMPARISON OF COMPOUND Z WITH SCHACHTER'S COMPOUND A.

Some properties of Compound Z and Schachter's Compound A are given below (Table 9). It would appear from the melting-points and analyses that the two compounds were different, although in view of Schachter's doubts as to the homogeneity of his material this is not certain. No sample of Schachter's compound was available for direct comparison in Edinburgh.

	Schachter's A	Klyne's Z
M.p.	190-192°	205-207°(corr.)
Solvents used	Aqueous acetone, then benzene	Aqueous ethyl alcohol
Analytical results	C, 77.5, 77.4 74.6	C, 79.5, 79.8, 80.4, 79.8
	H, 11.3, 11.2 11.0	H, 10.3, 10.1, 10.4, 10.2
Digitonin	Ppt.	Ppt.
Millon's test	Negative	Negative
Zimmermann test	Negative	Positive(slow)
Tetranitromethane	No colour	No colour

3, 16. DISCUSSION OF THE STRUCTURE OF COMPOUND Z. COMPARISON WITH Δ^{16}-ALLOPREGNENE-3(β)-OL-20-ONE.

Evidence

The elementary analyses and molecular weight determinations on Compound Z suggest that it probably has the molecular formula $C_{21}H_{32}O_2$. The elementary analyses of p-toluidine Z sulphate and of potassium Z sulphate are consistent with this (assuming that the potassium salt has one molecule of water of crystallisation).

The formation by Z of a mono-acetate ($C_{23}H_{34}O_3$), and/

and the precipitation with digitonin indicate a monohydroxy steroid with the hydroxyl group probably in the 3(β) position.

The slow formation of a purple colour in the Zimmermann test indicates the presence of a $COCH_2$ grouping. The failure to obtain a semicarbazone from Z appears at first sight at variance with this, but together with the non-formation of a yellow colour with tetranitromethane provides some negative evidence for assuming the presence of an α-β unsaturated ketonic grouping, C=C-C=O. Many αβ unsaturated ketones either fail to react or react in an anomalous manner with semicarbazide (Allen and Blatt, 1943; Meyer, 1938, gives many references at p. 544).

Compounds containing an olefinic double bond conjugated with a carbonyl group do <u>not</u> give a yellow colour with tetranitromethane (Ruzicka et al., 1929; Hurd, 1938). We have confirmed in this laboratory that progesterone (Δ^4pregnene-3,20-dione) and Δ^4 cholestene-3-one do <u>not</u> give colours with tetranitromethane.

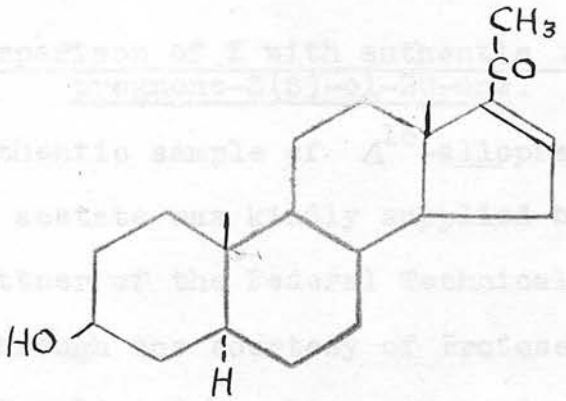
Suggested/

The properties of Compound Z and its acetate do not agree with those of any compound hitherto isolated from mare's urine.

Suggested Structure

All the above evidence led to the hypothesis that Z was a pregnenolone or allopregnenolone in which the hydroxyl group was in the 3(β) position, and the keto group and the olefinic double bond were conjugated.

A study of the literature showed that the only known compound of this type whose physical properties agreed with those of Z was Δ^{16}-allopregnene-3(β)-ol-20-one (IV), obtained by Marker et al. (1940,b) from dihydro-pseudo-tigogenin diacetate.



IV

Δ^{16}-alloPregnene-3(β)-ol-20-one

The properties of Z and of this allo-pregnenolone (as given by Marker) are compared in Table 10.

Table 10.

Comparison of Z and Δ^{16}-allopregnene-3(β)-ol-20-one.

	Z	Δ^{16}-allopregnene-3(β)-ol-20-one
Free hydroxy compound	Leaflets from aq. EtOH m.p.205-207° (corr.)	Crystals from dil. MeOH or ether m.p. 202-204°
Acetate	Hexagonal leaflets from aq. EtOH m.p. 163-165° (corr.)	Crystals from MeOH m.p. 162-164°

Comparison of Z with authentic Δ^{16}-allopregnene-3(β)-ol-20-one.

An authentic sample of Δ^{16}-allopregnene-3(β)-ol-20-one acetate was kindly supplied by Professor Pl.A. Plattner of the Federal Technical High School, Zurich (through the courtesy of Professor T. Reichstein of Basel) and has been compared with Compound Z acetate.

The determination of mixed melting-points with Z acetate and Plattner's acetate was difficult, since the melting-points of these compounds are dependent on the degree of crushing to which they have been subjected. This behaviour is fairly common/

common among steroids; cf. Reichstein, 1936 (Substance Fa), 1937 (dehydrocorticosterone) and private communication below.

Table 11.

Mixed melting-points. Z Acetate and Plattner's Acetate.

	Z Acetate	Plattner's Acetate	Mixture
1st set Edinburgh average crushing	162-163.5	162-164	159-163 158-162 158-161
2nd set Basel very thorough crushing	152-165	152-164	151-165
3rd set Edinburgh slight crushing	163-165	166-167.5	160.5-165.5

Note. 1. In all the melting-points done in Edinburgh, the substances softened 5-8° below the figures given above. It would appear that Professor Reichstein includes this softening in his melting range (see Basel m.p's).

2. Professor Reichstein's comment on the results of the 1st set of melting-points was as follows (letter of 19th June, 1946): "In my opinion a depression of mere 3-4° leaves a good amount of uncertainty since, as a rule, more pronounced depressions are observed. Quite often a depression may be caused by more intense grinding. If a sample used for a mixed melting point is subjected to severer grinding then the single/

single constituent before melting an apparent depression may be observed. If you would let me have a 1/2 to 1 mg. sample of your acetate (not the free hydroxy-ketone) I would have the mixed melting point checked in my laboratory. Mixed melting points of the free hydroxyketones are much less reliable than those of the acetates....."

The second set of melting-points was done in Professor Reichstein's own laboratory - for which I am much indebted to him.

Z acetate and Plattner's acetate behaved in precisely the same way in the following colour tests - concentrated sulphuric acid, Liebermann-Burchardt, tetranitromethane, Zimmermann ketosteroid.

Conclusion.

It may therefore be said that Z is probably, but not certainly, Δ^{16}-allopregnene-3(β)-ol-20-one.

Further Work Proposed.

When further supplies of Z are available the following experiments will be attempted. Products marked x could be used for comparison with authentic materials by mixed melting-points.

1. Formation of Ketonic Derivatives.

Oxime [x] or 4-phenylsemicarbazone.[x]

Some αβ-unsaturated ketosteroids form these derivatives normally, e.g. oximes of progesterone (Butenandt/

(Butenandt, Westphal and Hohlweg, 1934), testosterone (Butenandt and Hanisch, 1935), Δ^{16}-allopregnene-3, 20-dione (Butenandt, Mamoli and Heusner, 1939); 4-o-tolylsemicarbazone of cholestenone (Rosenheim and Webster, 1943).

2. Ultra-violet Absorption Spectrum.

The presence or absence of the 240 mμ band would indicate the presence or absence of the αβ-unsaturated ketonic group.

3. Catalytic Hydrogenation.

(a) With an Adams platinum catalyst both carbonyl and olefinic double bonds, if present, would be reduced. If Z is the allopregnenolone discussed above, the hydrogenation product would be allopregnane-3(β),20(β)-diol.[x] In any case the total hydrogen uptake would be very valuable information.

(b) With a palladium-barium sulphate catalyst the saturated ketone (allopregnane-3(β)-ol-20-one [x] ?) might be obtained (cf. Marker, 1940, c).

4. Chromic acid oxidation.

This might give a diketone (Δ^{16}-allopregnene-3,20-dione?) [x]

5. /

5. Polarographic Examination of the Girard Derivative.

This examination, which requires less than 1 mg. of material, might be of value when the technique has been applied to a wider variety of known compounds (Wolfe et al. 1940; Barnett et al., 1946).

PART 4.

THE ISOLATION AND STUDY OF Y SULPHATE AND OF COMPOUND Y.

4, 1. THE ISOLATION OF POTASSIUM Y SULPHATE

The combined 'water-insoluble' fractions from five batches of late pregnancy urine, on treatment in the usual way with 98% acetone, behaved in a very different manner from previous batches. Potassium Z sulphate was not obtained but a new compound, potassium Y sulphate, separated from the 98% acetone extracts on heating.

Experiment 016.

The 'water-insoluble' fraction (19.4 g.) from batches 09-012 and B13 (270 l.) was extracted with 98% acetone 11 times (A, 100 ml.; B-H, 200 ml. each; J-L, 100 ml. each). Each extract was filtered hot; the insoluble material was used for the next extraction, and the filtrate was cooled. In most cases the hot filtrate deposited some solid on cooling. These solid fractions (A-H/1) were separated by filtration or decantation, and the cold filtrates were heated in order to concentrate them.

In the case of the first five extracts, a flocculent precipitate (A-E/3) separated rapidly as soon as the temperature of the solution came near its boiling-point. (This precipitation was not due to the concentration of the solution by evaporation). The precipitates did not disappear when the solutions were cooled. This is not, therefore/

therefore, a case of a substance less soluble in the hot than the cold solvent. No explanation of this strange separation can be offered. After cooling the precipitates were filtered (A-E/3) and soon proved to be a sulphate different from that of Z (see next section). The filtrates were concentrated to small volume and deposited fairly crisp white precipitates (A-L/5). These were filtered off and their mother liquors all combined and evaporated to dryness (A-L/7).

These operations are outlined in Table 12 in flow sheet form. Note that letters A-L refer to successive extracts, numbers (1, 3, 5, 7, 8) to various fractions from each extract).

Some other observations on this fractionation are reported in Part 5, section 5.

Table 12. /

Table 12.

Isolation of Potassium Y Sulphate.

WATER-INSOLUBLE FRACTION (19.4 g.)

Successively extracted with 98% acetone (A, 100 ml.; B-H, 200 ml. each; J-L, 100 ml. each)
Each extract filtered hot.

Hot Filtrate

Cooled to room temp.
Solid filtered off

Solid used for next extraction

Insol. material from final extraction (L/8; 4.05 g.)

1st Cold Filtrate

Reheated to boiling.
In 5 cases ppt. separated.
Cooled and filtered again

Solid from 98% acetone on cooling (A-H/1; 0.9 g.)

2nd Cold Filtrate

Concentrated, cooled and filtered again

Solid from 98% acetone on reheating (A-E/3) $KYSO_4$ (1.1 g.)

3rd Cold Filtrate

Evaporated to dryness

Solid (A-L/7; ca. 10 g.)

Solid from 98% acetone on concn. (A-L/5; 1.6 g.)

4, 2. PROPERTIES OF POTASSIUM Y SULPHATE.

The Crude Sulphate

Fractions A/3 and B/3 from experiment 016 (see preceding section) were white crisp amorphous solids; fractions C/3-E/3 were white crystalline solids. All had melting-points (corr.) between 203-208° (slight browning; sintering ca 190°). Mixed melting-points between the fractions showed no depression, so all were combined (016/3).

Recognition of Compound 016/3 as different from Potassium Z Sulphate.

016/3 could not be recrystallised from water (contrast $KZSO_4$). On treatment with p-toluidine hydrochloride it gave a p-toluidine salt which differed considerably from p-toluidine Z sulphate in melting-point and solubility (see section 4,4).

Compound 016/3 was thus different from potassium Z sulphate and was named potassium Y sulphate.

In view of the difficulties which had been experienced in dealing with potassium Z sulphate, it was decided to transform the bulk of potassium Y sulphate into the p-toluidine salt. The only experiments carried out on potassium Y sulphate were the following.

Recrystallisation from ethyl alcohol.

A small quantity of potassium Y sulphate was recrystallised/

recrystallised twice from ethyl alcohol giving fine needles, m.p. 224-229° (corr.)(browning; sintering ca 210°). The salt is not much more soluble in hot ethyl alcohol than cold.

Behaviour with Boiling 98% Acetone.

An attempt was made to repeat the separation of potassium Y sulphate from 98% acetone solution on boiling, but without success.

Potassium Y sulphate (once recrystallised from ethyl alcohol, 13 mg.) was refluxed for 20 minutes with 98% acetone (10 ml.); nearly all the solid dissolved. The solution was filtered hot and the filtrate cooled; no precipitate appeared. The filtrate was brought to the boil and cooled several times, but no precipitate appeared.

4, 3. PREPARATION AND PURIFICATION OF p-TOLUIDINE Y SULPHATE.

This salt was prepared from crude potassium Y sulphate by treatment with p-toluidine hydrochloride in aqueous solution (as described for the Z salt on p. 61). The p-toluidine Y sulphate so obtained was apparently pure since recrystallisation from water did not raise its melting-point.

4, 4. PHYSICAL PROPERTIES OF p-TOLUIDINE Y SULPHATE

Melting Point (corrected)

181-183° (browning; softening at 180°)

After one month in vacuum desiccator, m.p. was 177-180° (corr.).

Crystalline Form

Very fine white needles (from water).

Specific Rotation

$[\alpha]_D^{20}$ = +16.1° ± 1° (C = 3 in ethyl alcohol)

Solubilities (very approximate)

Water 100°, 3.3 mg./ml.

0°, 1.0 mg./ml.

Ethyl alcohol 15-20°, readily soluble

Chloroform 15-20°, sparingly soluble

(contrast p-toluidine Z sulphate)

4, 5. ANALYSES AND CHEMICAL PROPERTIES OF p-TOLUIDINE Y SULPHATE

Analyses (Mr J.W.Minnis)

2.184 mg. substance gave 5.333 mg. CO_2, 1.769 mg. H_2O
2.073 mg. " " 5.019 mg. CO_2, 1.662 mg. H_2O

Sulphate determinations were carried out by acid hydrolysis and precipitation as $BaSO_4$.

2.931 mg. substance gave 1.433 mg. $BaSO_4$
2.925 mg. " " 1.448 mg. $BaSO_4$

Equivalent determinations (using Conway microburette; indicator phenolphthalein; W.K.)

8.941 mg. substance = 162.5 µl. 0.1056 N NaOH
9.885 mg. " = 179.5 µl. " "

	C	H	S	Equiv.
Found	66.6	9.06	6.71	521
	66.1	8.97	6.80	522
Calculated for				
$C_{21}H_{36}O.SO_4^-$ $CH_3.C_6H_4.NH_3^+$	66.2	8.93	6.32	507

Acid Hydrolysis - see next section.

4, 6. PREPARATION AND PURIFICATION OF COMPOUND Y.

Compound Y was prepared by acid hydrolysis of p-toluidine Y sulphate as described for Compound Z, except that 1 hour's heating at 100° was found to give a better yield than 2 hours' heating (88% as against 67%).

Compound Y was purified by repeated crystallisation from aqueous ethyl alcohol (approx. 2 vols alcohol: 1 vol. water).

4, 7. /

4, 7. PHYSICAL PROPERTIES OF COMPOUND Y.

Melting Point (corrected)

211-213°

This was not changed on drying to constant weight in vacuo at 80° and 110°.

Crystalline Form

Needles from aqueous ethyl alcohol.

Specific Rotation

$[\alpha]_D^{20} = +2° \pm 2°$ (C = 0.87 in ethyl alcohol)

Solubilities

Ethyl alcohol (cold) readily soluble

Ethyl alcohol (2 vols.) and water (1 vol.) (b.p.) 17 mg./ml.

Ethyl alcohol (2 vols.) and water (1 vol.) (0°) 7 mg./ml.

Sublimation

Y sublimed readily without decomposition at 180°/0.15 mm.

4, 8. ANALYSES AND CHEMICAL PROPERTIES OF COMPOUND Y.

Analyses

All by Mr J.W.Minnis.

(1) Material sublimed at 180°/0.06-0.1 mm.

1.875 mg. substance gave 5.380 mg. CO_2, 1.902 mg. H_2O
2.264 mg. " " 6.461 mg. CO_2, 2.275 mg. H_2O

(2) /

(2) Material recovered from the acetate and dried to constant weight at 110° over P_2O_5 in vacuo.

2.377 mg. substance gave 6.785 mg. CO_2, 2.377 mg. H_2O
2.807 mg. " " 8.036 mg. CO_2, 2.774 mg. H_2O

		C	H
Found (1)		78.3	11.35 %
		77.9	11.24 %
Found (2)		77.9	11.19 %
		78.1	11.06 %
Calculated	$C_{21}H_{36}O_2$	78.7	11.32 %
	$C_{21}H_{34}O_2$	79.2	10.76 %

Molecular Weight Determinations

0.312 mg. substance in 13.400 mg. camphor, D = 4°
0.693 mg. " 7.933 mg. " , D = 15°

M.W. found 233 ± 30, 233 ± 8.

The calculated molecular weight for $C_{21}H_{36}O_2$ is 320.

Compound Y is obviously a steroid,[x] for which a molecular weight of approximately 230 is impossible. Equivalent determinations on p-toluidine Y sulphate are consistent with the formulation of Y as $C_{21}H_{36}O_2$. It is therefore reasonable to assume that the compound behaves abnormally when dissolved in camphor.

Loss in Weight on Drying.

Three samples of Compound Y, previously dried in a vacuum desiccator at room temperature, when dried to constant weight over P_2O_5 in vacuo at 110° gave/

x See reactions (pp. 95-96)

gave the following results.

Losses in weight 3.03, 2.70, 2.75%

Calculated loss in weight for a hemi-hydrate

$C_{21}H_{36}O_2, \frac{1}{2}H_2O \longrightarrow C_{21}H_{36}O_2$

is 2.82%.

It thus seems probable that Compound Y crystallises from aqueous alcohol with one-half molecule of water of crystallisation, which is lost at 110°.

Reactions

Sodium Fusion Test for Elements.

This test showed that Y contains no nitrogen, sulphur or halogens.

Catalytic Hydrogenation

(This was carried out in the Chemistry Department of the University of Glasgow, thanks to the courtesy of Professor J.W.Cook, F.R.S.).

Compound Y absorbed no hydrogen at all on shaking with Adams platinum oxide catalyst for 2 hours in acetic acid solution.

Reaction with Digitonin

Y (in 90% ethyl alcohol (0.2 ml.) was treated (0.4 mg.) with digitonin (0.2 ml. 1 % solution in 90% ethyl alcohol). A precipitate first appeared after 30 minutes standing and increased in quantity during the next 40 minutes.

Colour/

Colour Tests

These were carried out as for Compound Z (see pp. 70-71). Results were as follows.

Concentrated sulphuric acid	Within 1 min. an orange red colour with a very slight green fluorescence developed. After 20 min. the colour was orange and the fluorescence had increased.
Liebermann Burchardt test	Conc. sulphuric acid layer, immediately deep crimson; chloroform layer colourless.
Tetranitromethane	No colour.
Zimmermann ketosteroid test	No colour.
Millon's Test	No colour.

4, 9. COMPOUND Y DIACETATE

Preparation

The diacetate of Compound Y was prepared by the method described for Z(on p. 73), and purified by chromatographic analysis and subsequent recrystallisation from aqueous methyl alcohol.

Purification by Chromatography

A representative chromatographic separation was carried out as follows.

Crude Y diacetate (257 mg. brown gum) was dissolved in 20% benzene; 80% pentane and run on to a column of aluminium oxide (20 x 80 mm., 20 g.) prepared/

prepared as before. The column was then eluted with the following solvents (15 ml. portions).

Fraction	Element	Eluate
1-6	80% pentane:20% benzene	Traces
7-11	50% pentane:50% benzene	Traces
12-19	Benzene	Crystalline material, m.p. <150° used for subsequent chromatogram (80 mg.)
20-25	"	Crystalline material, m.p. 156-160°, good Y diacetate (30 mg.)
26	95% benzene, 5% ether	As from fractions 12-19 (60 mg.)
27	90% benzene, 10% ether	
28-34	80% benzene:20% ether	
35-36	" "	Negligible traces
37-43	50% benzene:50% ether	
44-48	ether	

All the above fractions were perfectly white. The coloured impurities only started to move down the column when 50% ether:50% acetone was used as eluent.

Certain fractions in the group 12-19 were washed with ice-cold pentane in an attempt to remove impurities and improve the melting-point. This result was achieved, but so much material was washed away that the process was not worth while.

Physical/

Physical Properties

Melting Point (corrected)

159.5°-160.5°

Crystalline Form

Glistening plates from aqueous methyl alcohol.

Specific Rotation

$[\alpha]_D^{19}$ = -32° ± 0.6° (C = 3 in chloroform)

Solubility

Very approximate

50% methyl alcohol: 50% water

at b.p. 8 mg./ml.

at 0° 4 mg./ml.

Analyses

On material dried to constant weight[x] at 80° in vacuo over P_2O_5 (Mr J.W.Minnis)

2.366 mg. substance gave 6.416 mg. CO_2, 2.161 mg. H_2O
3.345 mg. " " 9.124 mg. CO_2, 3.007 mg. H_2O

		C	H
Found		74.0	10.22 %
		74.4	10.06 %
Calculated	$C_{25}H_{40}O_4$ pregnanediol diacetate	74.2	9.96 %
	$C_{25}H_{38}O_4$ pregnenediol diacetate.	74.6	9.51 %
	$C_{23}H_{38}O_3$ pregnanediol monoacetate	76.2	10.51 %

x Loss in weight 0.3% (negligible)

Hydrolysis

An attempt to hydrolyse Y diacetate with cold methyl alcoholic potash proved unsuccessful.

To Y diacetate (15 mg.) in methyl alcohol (2 ml.) was added potassium hydroxide (9 mg. = 4 moles) in methyl alcohol (0.4 ml.) and the mixture was left to stand at room temperature for 52 hours. At the end of this time the colourless solution was diluted with ether (50 ml.) and this was washed with water (3 x 5 ml.) to remove alkali and taken to dryness. The residue had m.p. 158-166°(corr.) and presumably contained much unhydrolysed diacetate.

Hydrolysis was achieved with boiling methyl alcoholic potassium hydroxide. Y diacetate (40 mg.) in methyl alcohol (4 ml.) was refluxed on the boiling water-bath for 2 hours with potassium hydroxide (90 mg. = 16 moles) and the product worked up as before. The crude product (38 mg.) had m.p. 193-199° (corr.) (after softening from 170°).

4, 10. Y KETONE

Preparation and Purification

Compound Y was oxidised by chromium trioxide in acetic acid to a substance named 'Y ketone'.

Compound Y (18 mg.) in glacial acetic acid (5 ml.) was treated with chromium trioxide (12 mg.) in 98% acetic acid (0.6 ml.). The red colour of the chromium trioxide rapidly changed to a dull brown but no further change occurred on standing. After 22½ hours at room temperature water (100 ml.) was added to the reaction mixture and the resulting precipitate was filtered, washed free from acid and dried (16.5 mg.). The product was recrystallised twice from a mixture of equal volumes of acetone and water.

Physical Properties

Melting Point (corrected)

171-173° (softening 169°)

Determined on Kofler apparatus.

Crystalline Form

Small plates from aqueous acetone.

Analyses /

Analyses.

On material dried 2 hours at 80° in vacuo over P_2O_5 (Mr J.W.Minnis).

1.852 mg. substance gave 5.436 mg. CO_2, 1.744 mg. H_2O
2.011 mg. " " 5.900 mg. CO_2, 1.833 mg. H_2O

	C	H
Found	80.1	10.53 %
	80.1	10.20 %
Calculated $C_{21}H_{32}O_2$ pregnanedione	79.7	10.19 %
$C_{21}H_{34}O_2$ pregnanolone	79.2	10.76 %

4, 11. Y KETONE SEMICARBAZONE

Y ketone on treatment with semicarbazide hydrochloride and sodium acetate in the usual way gave a mono-semicarbazone.

Y ketone (4.5 mg.) in ethyl alcohol (5 ml.) was treated with semicarbazide hydrochloride (3.2 mg., 2 moles) and hydrated sodium acetate (4.5 mg., 2.3 moles) in water (0.5 ml.) and allowed to stand at room temperature for 3 days. After 18 hours' standing further semicarbazide hydrochloride (6.4 mg.) and hydrated sodium acetate (10 mg.) were added to ensure a large excess.

After 3 days most of the alcohol was evaporated, and the product was precipitated with water, filtered, washed and dried.

After one recrystallisation from ethyl alcohol, the/

the product was a white powder, m.p. (corr.) 217-221° (browning), after softening 206-216°.

Analysis

1.396 mg. substance gave 0.143 ml. (corr.) nitrogen at 15° and 756 mm.

Found	12.1% N
Calculated for mono-semicarbazone of a ketone $C_{21}H_{32}O_2$	11.1% N
Calculated for di-semicarbazone of a ketone $C_{21}H_{32}O_2$	19.5% N

4, 12. DISCUSSION OF THE STRUCTURE OF COMPOUND Y. COMPARISON WITH 'URANEDIOL'

Evidence and Deductions

The elementary analyses of Compound Y and of its diacetate, and its resistance to catalytic hydrogenation indicate that it is a saturated dihydroxy compound $C_{21}H_{36}O_2$. The elementary analyses and the neutralisation equivalent of p-toluidine Y sulphate are in agreement with this formula.

The behaviour of compound Y in the usual colour tests and its precipitability with digitonin indicate that it is a steroid, one of the two hydroxyl groups probably being in the 3(β) position. It is probably an isomer of pregnanediol.

Y ketone forms a mono-semicarbazone and it must therefore be assumed either that Y ketone is a diketone/

diketone one of whose carbonyl groups is unreactive, or that it is a hydroxyketone ($C_{21}H_{34}O_2$) and that one of the hydroxyl groups of Compound Y is resistant to oxidation. It is impossible to test these assumptions further at present since no material is available.

If Y ketone is a diketone, the unreactive carbonyl group may be at C-11 (cf. Steiger and Reichstein, 1937; Reichstein and Shoppee, 1943 (at p.370)). The objection may be raised that C-11 hydroxyl groups in the adrenal steroids and their derivatives can be acetylated only with great difficulty or not at all (Reichstein and Shoppee, reference above), while Compound Y readily gives a diacetate. However, Reich and Reichstein (1943) pointed out that an 11(α) hydroxyl group should be less sterically hindered than the 11(β) groups found in the adrenal steroids; they showed that the 11-hydroxy-cholanic acid methyl ester obtained from the corresponding ketoester by catalytic hydrogenation with Raney nickel could be acetylated slowly.

It would be idle to speculate further in this way until further information about Compound Y is available, and in particular, until Y has been converted into some known substance.

Comparison/

Comparison with Known Substances

Y and its derivatives do not resemble any known pregnanediol or allo-pregnanediol and its derivatives. They do, however, resemble rather closely the 'uranediol' of Marker et al. (1938,f) and its derivatives. All known[x] pregnanediols and allo-pregnanediols together with 'uranediol' and Compound Y) and the relevant derivatives are listed in Table 13.

Table 13 /

x Abstracts searched - Chemisches Zentralblatt to 1938 inclusive; British Chemical Abstracts A, 1939 to date.

Table 13.

Pregnanediols, Allopregnanediols, Uranediol and Compound Y.

Melting Points

	Diol	Diacetate	Dione	Dione semi-carbazone
Pregnane				
3α 20α	236	179		
3α 20β	231-4	110	118	257 dec.(d)
3β 20α	182	141		
3β 20β	174-6	111		
3α 21	205-6		(b)	-
3β 21	164-6	76-9	(b)	-
Allopregnane				
3α 20α	248	182-3		
3α 20β	207	124	200	325(d)
3β 20α	215	166-8		
3β 20β	192-4	142-3		
3β 17α	221-2	(a)	-	-
3β 17β	174 or 187	(a)	-	-
3β 21	183-4	135	(b)	-
Urane				
3β 11(?)	210	160	177.5	245 dec.(M)
Compound Y	211-3	159.5-160.5	171-3	217-221 dec.(M)

(a) The 3,17-diols would give mono-acetates and Y cannot be identical with either of them.

(b) The 3,21 diols would give keto-acids on oxidation, and Y cannot be identical with any of them.

(d) Disemicarbazone.

(M) Monosemicarbazone

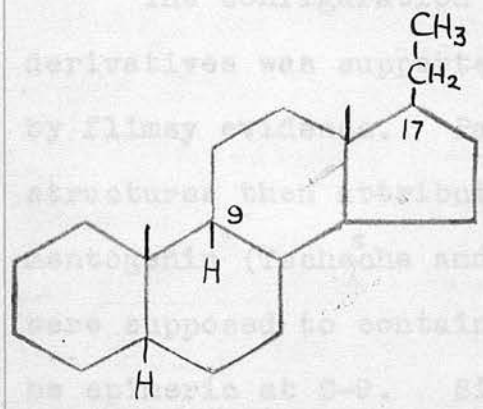
It/

It has so far been impossible to obtain samples of Marker's 'uranediol' and derivatives for comparison, and as far as can be seen from the literature no one except Marker and his colleagues has ever handled a 'urane' derivative.[x]

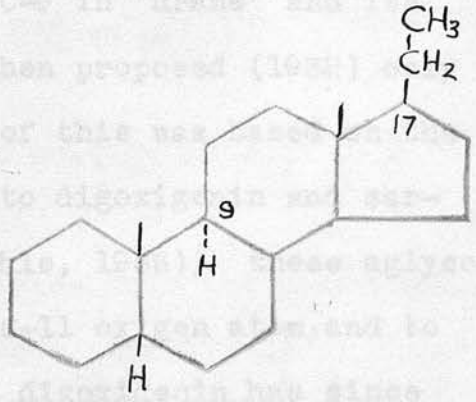
It should be noted that Marker's 'uranediol', which analyses well for a diketone, forms only a monosemicarbazone, as does Y ketone.

Marker's Evidence for the Structure of Urane and Its Derivatives - and Criticism thereof

Marker (1938,b) transformed his 'uranediol' and 'uranetriol' (also from mare's pregnancy urine) into the corresponding ketones 'uranedione' and 'uranetrione'. From each of these he obtained by Clemmensen reduction a hydrocarbon 'urane' ($C_{21}H_{36}$) m.p. 124° which was certainly different from pregnane and allopregnane. By a complex series of reactions 'uranetrione' was transformed into a mixture of 'uranedione' and pregnane-3,20-dione.



V.
Urane



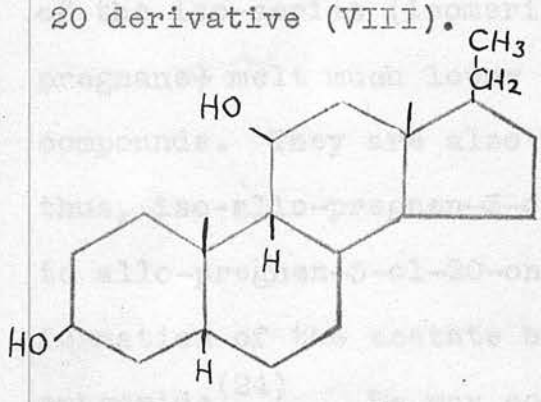
VI.
Pregnane

x The only references giving details of urane derivatives are Marker et al. (1938,b,e,f,h; 1939,d; ✱1944 (patent))

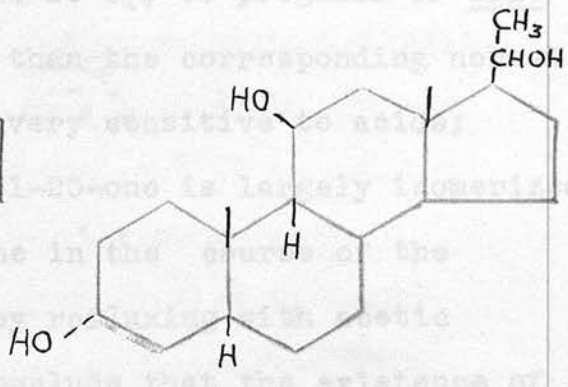
Possible Isomerism at C-9.

Marker (1938,b) suggested that 'urane' was a stereoisomer of pregnane and proposed for it the structure (V) which is epimeric with pregnane(VI) at C-9.

'Uranediol' was presumed to be a 3(β),11 derivative (VII), since it gave a precipitate with digitonin and its oxidation product, 'uranedione' contained an unreactive keto group (cf. the adrenal steroids.) 'Uranetriol' was presumed to be a 3(α),11, 20 derivative (VIII).



VII
Urane-3(β),11-diol



VIII
Urane-3(α),11,20-triol

The configuration at C-9 in 'urane' and its derivatives was supported when proposed (1938) only by flimsy evidence. Part of this was based on the structures then attributed to digoxigenin and sarmentogenin (Tschesche and Bohle, 1936); these aglycons were supposed to contain a C-11 oxygen atom and to be epimeric at C-9. Since digoxigenin has since been shown to have a C-12 and not a C-11 oxygen atom (Hoehn/

(Hoehn and Mason, 1938; Mason and Hoehn, 1938,a,b; Steiger and Reichstein, 1938,a), this part of Marker's evidence falls to the ground.

Possible Isomerism at C-17.

Marker's evidence (1938,b) against the hypothesis that 'urane' compounds might be epimeric with pregnane at C-17 is inaccurate, and would be insufficient even if it were accurate. He says (at p. 1064): "Isomerization at C_{17} seems very unlikely since the known compounds of the iso-series (isomeric at C_{17} to pregnane or allo-pregnane) melt much lower than the corresponding normal compounds. They are also very sensitive to acids; thus, iso-allo-pregnen-3-ol-20-one is largely isomerized to allo-pregnen-3-ol-20-one in the course of the formation of the acetate by refluxing with acetic anhydride[24]. We may conclude that the existence of urane compounds is not due to isomerism at C-17."

[(24) Butenandt and Fleischer. Ber. 70,96 (1937). This article gives references to earlier papers on compounds of the iso-series].

The following comments must be made on this statement:

(1) Melting points - When Marker made the statement, only five 17-iso compounds were known. One of these, Δ^5-17-isopregnene-3(β)-ol-20-one acetate, melts higher than the corresponding 17-n compound (171° against/

against 147°, (Butenandt and Fleischer, 1937). Marker's statement is therefore untrue. Even if all five 17-iso compounds had melted lower than the corresponding 17-n compounds, this number would have been quite insufficient to permit generalisation.

(2) Isomerisation by acid - The statement that 17-iso compounds are 'very sensitive to acids' had virtually no basis at all when Marker made it.

In 1938 the only reported cases in which 17-iso compounds had been submitted to acid conditions were two acetylations (in neither of which isomerisation took place) and one oxidation by chromic acid in glacial acetic acid (in which sone 17 n- compound was obtained). For references see Appendix C.

Marker's statement that 17-iso- -allo-pregnen-3-ol-20-one is largely isomerised by refluxing with acetic anhydride appears to be based on a mis-translation of the word 'umestern' in the paper of Butenandt and Fleischer (1937). These authors say (p. 98): "Um das freie 17-iso-Pregnenolon darzustellen war der Weg über das Acetat nicht gangbar, da dieses sich nicht ohne Umlagerung verseifen oder umestern liess".

This mistake in translation is surprising, since Butenandt and Fleischer in the same paragraph made some calculations regarding specific rotations which were based on the assumption that there was no isomeration/

isomerisation during acetylation, and which were subsequently proved correct.

Appendix C (pp. 159-162) summarises the data now available on 17-iso-pregnane and 17-iso-allopregnane compounds in which a hydrogen atom is attached to C-17.

Professor Marrian has suggested that if urane is a stereoisomer of pregnane and allopregnane, then on biogenetic grounds, isomerism at C-17 is much more likely than isomerism at C-9.

Conclusion

Compound Y is a saturated dihydroxy steroid, isomeric with pregnanediol ($C_{21}H_{36}O_2$). One hydroxyl group is probably in the 3(β) position, the other may be in the 11 position. Compound Y may be identical with Marker's 'uranediol'.

Further Work Proposed on Compound Y and Derivatives.

1. Comparison with Uranediol and Derivatives

Mixed melting-points and colour tests (if material can be obtained from Dr Kamm).

When more of Compound Y is available, the following experiments will be tried.

2. Clemmensen Reduction of Y Ketone.

This will be given first priority since if pregnane or allopregnane were formed, this would show that/

that Y is a derivative of one of these well-known hydrocarbons and would greatly simplify further work.

3. Formation of further Ketonic Derivatives from Y Ketone.

Dinitrophenylhydrazone (cf. Marker's mono-derivative from uranedione). Oxime.

4. Catalytic Hydrogenation of Y Ketone.

The hydrogen uptake would show whether Y ketone is a diketone $C_{21}H_{36}O_2$ or a hydroxy-ketone $C_{21}H_{34}O_2$.

5. Subsequent Work.

This would depend on the results of the above. If Y ketone gives pregnane or allopregnane on reduction, the next step would be to try and locate the two oxygen atoms; if the present very slight evidence in favour of positions C-3 and C-11 is supported by subsequent work, the synthesis of pregnane-3,11-dione or allopregnane-3,11-dione, would be necessary.

If Y ketone gives some hydrocarbon other than pregnane or allopregnane (e.g. 'urane'), a major problem in steroid chemistry appears. It would be worth considering the synthesis of C-17 iso compounds without a carbonyl group at C-20.

PART 5.

OTHER EXPERIMENTS ON MARE'S URINE CONJUGATES.

5, 1. SOME QUANTITATIVE DATA ON THE STANDARD METHOD FOR WORKING-UP URINE.

Summary of Results

The number of batches of urine worked up by the standard method of Section 2,7 (pp.35-42) is hardly sufficient to permit of anything but rough generalisations about the proportions in which the various fractions occur. The data available at present are collected in Table 14 (next page) since they may be of value in planning future work.

Notes on Table 14.

1. Many weights are approximate since it is difficult to remove all traces of butyl alcohol from distillation residues.
2. Gaps in the table indicate that the fraction concerned was not weighed.
3. The 'potassium acetate soluble' fractions were worked up as described in Section 5, 2.

Study of Butyl Alcohol Extractions and of Removal of Acidic Material

Batches 01 and 02 were extracted four times with butyl alcohol in the normal way, but the four extracts were separately washed with alkali and water and taken to dryness, so that the proportions of material removed in each extraction might be ascertained.

The/

Table 14

Results of Standard Method for Working Up Urine. (All weights of fractions are given in mg. per litre urine)

Batch	Weeks before foaling	Volume l.	Total neutral frn. mg./l.	Potassium acetate sol. frn. mg./l.	Potassium acetate insol.frn. mg./l.	Water soluble frn. mg./l.	Water insoluble frn. mg./l.
N11	11	21	630				170
24	8-9	15	470		370		140
36	0-3	22	560		390		210
01-3	11-14	52	300	25	240	190	50
4-5	9-10	36	310	35	150		
6	8	18	320	35	280	160	90
9	2	18	810	55	340		90
10	1	17	520	60	450		210
11	1	18	300	80	260		70
12	1	18	210	20	140		100
B13	few	200	360	40	230		55
Mean			440	40	280		120
Standard Deviation			170	20	100		60
No. of Observations			11	8	10		10

The acidic material from each extract was obtained by acidifying the alkali washings to Congo red with hydrochloric acid, and extracting three times with butyl alcohol (1/6 volume each time). The butyl alcohol extracts containing the acidic material were washed with water and evaporated to dryness. The residues were dark brown or black tars with most repulsive smells, suggestive both of phenols and of lower fatty acids (e.g. valeric). Some of these fractions crystallised in part on long standing.

The weights of the neutral and acidic fractions thus obtained are given in Table 15 . Note that these neutral fractions had still to be put through the hydrochloric acid-ether treatment. Weights of the acidic fractions are very approximate, since these contain readily-volatile material.

Table 15.

Weights of Neutral and Acidic Material obtained in Successive Extractions.

All weights in mg. per litre urine.

	Neutral		Acidic	
	Batch 01	Batch 02	Batch 01	Batch 02
1st BuOH extract	430	300	1400	700
2nd " "	210	210	1000	1050
3rd " "	150	120	1050	800
4th " "	200	120	600	700
Total	1390	750	4050	3250

Conclusion - Extraction of urine four times with butyl alcohol (1/4 volume each time) achieves nothing like complete removal of butyl alcohol soluble material.

5, 2. THE POTASSIUM ACETATE SOLUBLE FRACTION. ISOLATION OF p-TOLYL SULPHATE.

p-Tolyl sulphate was isolated from the potassium acetate soluble fraction obtained from mare's pregnancy urine by the standard method (Section 2,7) and characterised as its p-toluidine salt.

Extraction/

Extraction with Butyl Alcohol and Crystallisation from Methyl Alcohol.

The following experiments were carried out on the potassium acetate soluble fraction of Batches 01-3 (52 l. urine, collected 11-14 weeks before foaling).

The half-saturated potassium acetate solution, from which insoluble material had been removed by centrifuging (300 ml.), was extracted with butyl alcohol (4 x 100 ml.). The butyl alcohol extracts were washed with water (3 x 50 ml.) and evaporated to dryness giving a cream powder (1.3 g.). This material crystallised from hot methyl alcohol (10 ml.) to give an almost white granular solid (Fraction 01 L, 0.55 g.). This solid which was soluble in water gave no colour with Kober's reagent. A roughly quantitative Tollen's test showed that it contained about 1% glucuronic acid.

Hydrolysis

Part of 01 L (135 mg.) was hydrolysed by heating with aqueous hydrochloric acid (1M, 10 ml.) at 100° for 2 hours. The products were extracted with ether and the material extracted separated intp phenolic and non-phenolic fractions in the usual way. The phenolic fraction was an oil of the usual/

usual phenolic smell (47 mg.). It gave a blue-violet colour with aqueous ferric chloride (cf. p-cresol) and a positive Millon's test. The non-phenolic fraction was negligible in quantity (ca. 1 mg.).

The aqueous solution from the hydrolysis gave a strongly positive test for sulphate.

Formation of p-Toluidine Salt. Comparison with p-Toluidine p-tolyl Sulphate.

The remainder of fraction 01 L (330 mg.) was again recrystallised from methyl alcohol (3 ml.) giving 130 mg. pure white crystals, which did not melt up to 265°. This product was treated with an equal weight of p-toluidine hydrochloride in hot water (3 ml.). On cooling fine long needles (134 mg.) separated. These after recrystallisation from water had m.p. 163-164° (softening 152-158°). The mixed melting point with authentic p-toluidine p-tolyl sulphate of m.p. 161-164° was 158-162°.

Owing to lack of facilities at the time this part of the work was done, no elementary analyses were carried out on the p-toluidine p-tolyl sulphate isolated from urine as described above. Its identity cannot be considered as proven until analyses have been done, and further derivatives prepared.

Conclusion

All the above evidence suggests that the potassium acetate soluble fraction contains much potassium p-tolyl sulphate mixed with similar simple phenolic sulphates.

It is, of course, well known from the work of Baumann (1876,a,b; 1878-9) that mare's urine contains phenyl and p-tolyl sulphates. It is somewhat surprising that there is no mention of improved methods of extraction in the literature since this date.

The recent isolation by Lederer (1943) of p-ethyl phenol from the hydrolysates of mare's pregnancy urine suggests that p-ethyl phenyl sulphate is probably present in this fraction.

Further Work Proposed

The potassium acetate soluble fractions of all later batches were extracted with butyl alcohol as described above and the residues from the extracts kept for further work on these simple phenolic sulphates.

Probable Separation of p-Tolyl Sulphate from the Total Neutral Fraction

A crystalline substance which was shown to be probably impure potassium p-tolyl sulphate was obtained by accident from some batches of non-pregnant mare's urine, when a solution of the total neutral fraction/

fraction in hot methyl alcohol was cooled. See also p. 27 regarding similar experiences with some batches of pregnancy urine.

5,3. THE 'WATER-SOLUBLE' FRACTION

No pure compound has been separated from the 'water-soluble' fraction obtained by the standard method of Section 2,7.

Several unsuccessful attempts were made to obtain more potassium Z sulphate or to separate other materials from the 'water-soluble' fraction of Batch M12.

(1) Part of the material was taken up in a minimum of hot water; the solution on cooling gave only a very faint precipitate.

(2) The material was readily soluble in 98% acetone (except for a small residue). Concentration of the acetone solution yielded a small quantity of solid which was not potassium Z sulphate, and was not further investigated.

(3) The bulk of the material was put through the potassium acetate treatment, and the potassium acetate insoluble fraction put through Schachter's chloroform treatment. 98% acetone treatment of the product yielded a material which was apparently not potassium Z sulphate, and was not further investigated.

On/

On account of the difficulties experienced with the 'water-soluble' fraction of Batch M12, and lack of time, most subsequent 'water-soluble' fractions were not investigated, but made alkaline and stored for hydrolysis to obtain the oestrone which was probably present.

5, 4. INVESTIGATION OF HYDROLYSATES FROM 'WATER INSOLUBLE' AND 'WATER SOLUBLE FRACTIONS

Since Batches 01-3 failed to give any potassium Z sulphate on 98% acetone treatment, the conjugates present in the 'water-insoluble' and 'water-soluble' fractions were studied by hydrolysis and fractionation of the free hydroxy compounds so obtained. Batches 01-3 consisted of 52 l. urine, collected 11-14 weeks before foaling.

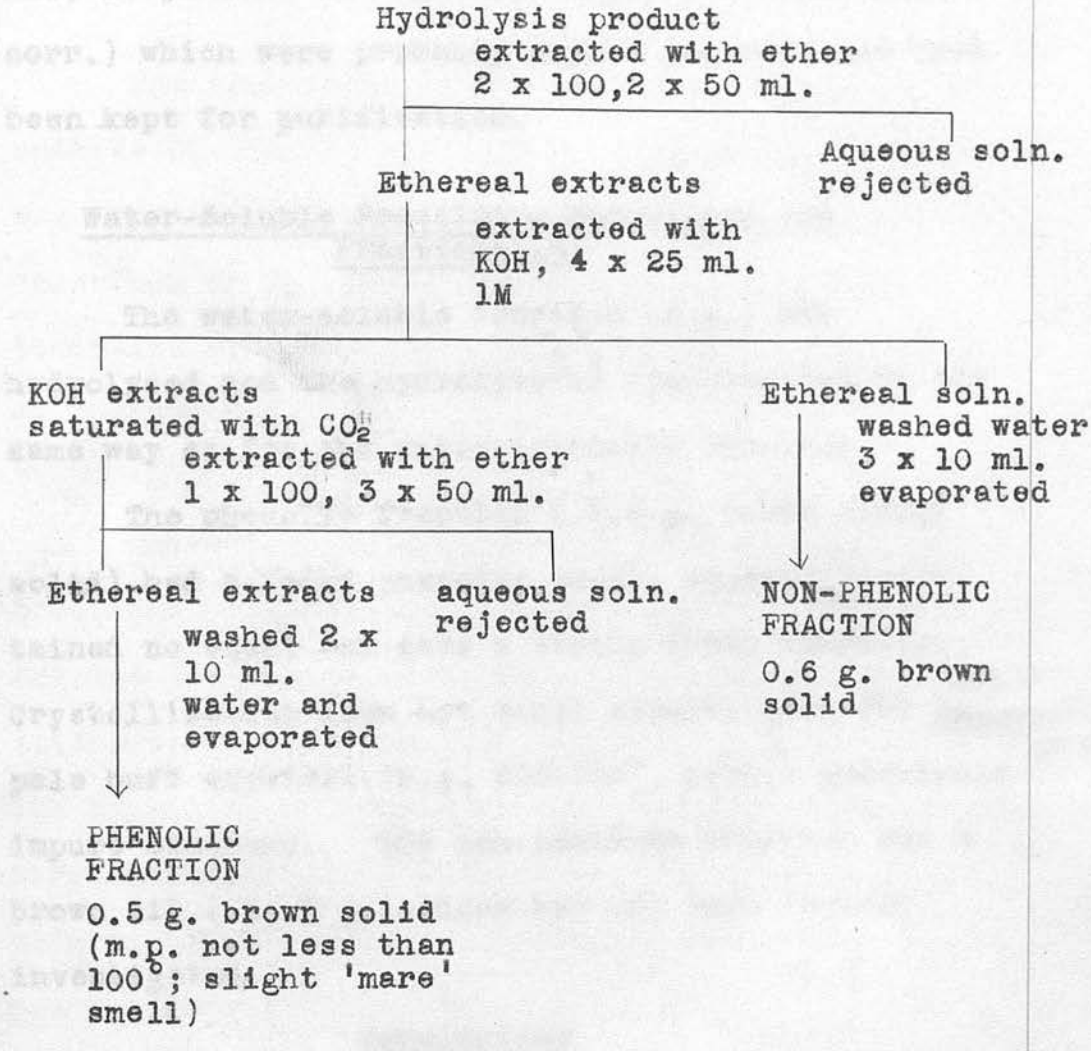
Water-Insoluble Fraction - Hydrolysis

The water-insoluble fraction (2.0 g.) was dissolved in water (200 ml.) and extracted with ether (3 x 50 ml.) to remove any free steroids present (only 50 mg. were so removed). Hydrochloric acid was added (18 ml. conc. acid, to make solution 1 N) and the mixture was heated at 100° for 2 hours. The solution became turbid and greenish; towards the end of the heating, a brown precipitate formed and the/

the mixture had a strong phenolic smell. The mixture was cooled and extracted as shown in Table 16

Table 16.

Fractionation of Hydrolysates from water-insoluble Fraction.



The phenolic fraction was dissolved in hot chloroform (50 ml.); on cooling and standing no precipitate separated. No appreciable quantity of equol was therefore present, since this substance separates readily from chloroform solutions of phenolic/

phenolic fractions if present (see Marrian and Beall, 1935).

The phenolic fraction gave a strong Kober reaction. When crystallised from ethyl alcohol (5 ml.) it yielded 160 mg. buff crystals (m.p. 226-230°, corr.) which were probably impure oestrone and have been kept for purification.

Water-Soluble Fraction - Hydrolysis and Fractionation

The water-soluble fraction (8 g.) was hydrolysed and the hydrolysates fractionated in the same way as for the water-insoluble fraction.

The phenolic fraction (2.5 g. brown sticky solid) had a faint phenolic smell, apparently contained no equol and gave a strong Kober reaction. Crystallisation from hot ethyl alcohol gave 570 mg. pale buff crystals (m.p. 225-235°, corr.) presumably impure oestrone. The non-phenolic fraction was a brown oil (ca. 2 g.) which has not been further investigated.

Conclusions

The results from this batch of urine, assuming that it is fairly typical of late pregnancy urine, suggest the following conclusions:

1. Water-soluble and water-insoluble fractions each contain phenolic and non-phenolic conjugates in roughly equal amounts.

2. /

2. Oestrogen sulphates are found in large amounts in both water-soluble and water-insoluble fractions.

5, 5. FURTHER OBSERVATIONS ON THE 'WATER-INSOLUBLE' FRACTION FROM WHICH POTASSIUM Y SULPHATE WAS OBTAINED.

The 'water-insoluble' fraction (19.4 g.) from batches 09-012 and B13 (270 l. late pregnancy urine) was fractionated by 98% acetone treatment as described on pp. 86-88 (Experiment 016). The isolation of potassium Y sulphate has been described on these pages.

Oestrogen and Glucuronic Acid Determinations

These determinations were carried out on the fractions from the 98% acetone treatment, and the results are tabulated below. Oestrogen determinations were kindly carried out by Miss M.F. Stevenson by the Kober method as modified by Stevenson and Marrian (unpublished work). Glucuronic acid determinations were made according to Hanson, Mills and Williams (1944).

Table17.

Oestrogen and Glucuronic Acid Determinations on Fractions from Experiment 016.

Fraction	Origin Weight Appearance and corr. m.p.(if taken)	% Oestrogen (by Kober test)	% Glucuronic Acid (by naphthoresorcinol test)
1	Separated from 98% acetone on cooling without concn. (0.9 g.) Crisp, fawn m.p. 204-208°	6	<1
3	Separated from 98% acetone on reheating and recooling, without concn. Largely $KYSO_4$ (1.1 g.) Crisp, white m.p. 203-208°	0	<1
5	Separated from 98% acetone after concn. (1.6 g.) Crisp, buff-white m.ps. 185-218°	11	<1
7	Obtained by taking 98% acetone mother-liquors to dryness (ca. 10 g.). Pale brown powder. m.p. 165-195°	13	<1
8	Matl. insol. in 98% acetone(4.05 g.) Greyish powder m.p. 215-240°	0	6

The following conclusions regarding the 'water-insoluble' fraction of the conjugates of pregnancy urine may be drawn:

(a) The conjugated oestrogens are present chiefly in those fractions which are most soluble in aqueous acetone (5 and 7 above).

(b) The glucuronides are contained entirely in the fraction (8), which is insoluble in 98% acetone.

(c) No oestrogen glucuronides are present.

Attempts to purify various fractions

Fraction 1 - This, although melting at approximately the same temperature as fraction 3 (crude $KYSO_4$), gave a depression on admixture with this. Fraction 1 could not be crystallised from water or from ethyl alcohol, and did not give a precipitate with p-toluidine hydrochloride in aqueous solution. The 98% acetone treatment was repeated in fraction 1, but no tractable product was obtained.

Fraction 5 - This could not be crystallised from water, and did not give a precipitate with p-toluidine hydrochloride in aqueous solution. The 98% acetone treatment was repeated on this fraction. A small quantity of white crystals were obtained which after two recrystallisations from ethyl alcohol gave small needles, corr. m.p. 254-259° (softening 241°). This compound has not been further investigated.

APPENDIX A.

SUGGESTIONS FOR FURTHER WORK.

1. Further Study of Compounds Z and Y.
2. Attempts to improve the Standard Method of extracting and isolating Compounds Z and Y.
3. Study of the Excretion of Compounds Z and Y throughout Pregnancy.
4. General Study of Conjugates throughout Pregnancy by Hydrolysis.
5. The Pure Chemistry of Organic Sulphates.
6. Study of New Methods for isolating Conjugates from urine.
7. General Study of Conjugates as such throughout Pregnancy.
8. Application of the work in Veterinary Medicine.

---

1. Further Study of Compounds Z and Y.

It is hoped to obtain more late pregnancy urine (200 l. or more) in the spring of 1947. If further supplies of Compounds Z and Y can be obtained, further chemical investigations will be carried out (as outlined on pp. 82-84 and 110-111) with a view to identifying the two compounds.

If Compound Y proves to be a derivative of some C_{21} hydrocarbon other than pregnane or allo-pregnene ('urane'?), it will be a discovery of first-class importance in steroid chemistry and much work will be necessary.

An attempt has been made to obtain samples of Marker's 'urane' derivatives through his co-worker Dr Kamm.

2. /

2. Attempts to improve the Standard Method of extracting and isolating Compounds Z and Y.

The Standard Method of Part 2, section 7 could undoubtedly be improved by a systematic study of each stage in turn with adequate controls, full use being made of additional knowledge about the physical and chemical properties of Z and Y sulphates from the first part of this proposed work. 200 Litres of 11th (last) month pregnancy urine would probably suffice for this study.

3. Study of the Excretion of Compounds Z and Y throughout Pregnancy.

It is suspected that Compounds Y and Z appear in the urine in large quantity only towards the end of pregnancy. A semi-quantitative study might be made taking ≡ 20 l. batches of urine from each of 6 mares, first in the non-pregnant condition, and then at each month during pregnancy. The material should be worked up by a standard method - presumably the present method as amended by the results of (2) above.

It would probably be worth while examining stallions' and geldings' urine (6 x 20 l. batches of each) for the presence of Compounds Z and Y at the same time.

4. General Study of Conjugates throughout Pregnancy, by Hydrolysis.

All the above work has been concerned with two compounds Y and Z which, it is suspected, are present in large quantities only at the end of pregnancy, and which are certainly accompanied by several (perhaps many/

many) related conjugates. Since so little is known about the properties of steroid sulphates and other conjugates, the general nature of the conjugates during pregnancy could best be started by a systematic examination of their hydrolysates. Again 6 x 20 l. batches of urine from different mares (non-pregnant and at each month of pregnancy) might be used. The urine should be extracted with butyl alcohol and acidic material removed in the usual way.[x] The total neutral fraction should then be hydrolysed by acid and the hydrolysis products fractionated into phenolic and non-phenolic, ketonic and non-ketonic etc. by the usual procedures. (Some preliminary experiments have been carried out by Mr J.K. Grant on these lines).

5. The Pure Chemistry of Organic Sulphates.

New methods for studying steroid conjugates in biological materials can only be based on a thorough knowledge of the physical and chemical properties of organic sulphates[xx] (and other conjugates), which does not exist at present.

Organic sulphates appear in many places in biological chemistry, and an adequate knowledge of their properties/

x Tests for oestrogen and glucuronide should be done on each fraction.

xx By 'organic sulphates' is meant half-esters of the type $R.SO_4^-B^+$ where B^+ is a cation and R a hydrocarbon radical.

ties would be valuable in many fields.

On a long-term basis this study should be given first priority, and as the author's preference is for pure organic chemistry he intends to devote the next 2-3 years to a study of organic sulphates on the lines indicated below.

Methods of Preparation.

Physical Properties - especially solubilities (using the rapid method of Klyne and Bell, 1946).

Chemical Properties - especially stability and conditions of hydrolysis. Later the use of the radio-isotope of oxygen to study the mechanism of hydrolysis.

Derivatives for Characterisation and Isolation, e.g. S-benzyl-pseudo-thiuronium salts, which appear promising (unpublished results).

Methods of Separation - e.g. attempts to use ion-exchange resins or partition chromatography.

The above work would be started with simple alkyl and aryl sulphates, and then extended to synthetic steroid sulphates.

(6) Study of new methods for isolating conjugates from urine.

The knowledge on the chemistry of sulphates gained in (5) should suggest manypossible lines of improvement in the methods for separation and isolation of urinary conjugates.

If successful, the ion-exchange or partition chromatography methods would represent major advances. Lesser, but valuable, advances would almost certainly result from a better knowledge of the solubilities of representative sulphates in a wide variety of solvents/

solvents. An example is the partition of p-toluidine salts between chloroform and water, which has been tried in a preliminary fashion (pp. 33-35).

The formation of derivatives by other functional groups present in the molecules of urinary steroid sulphates might be worth investigation (e.g. Girard or similar derivatives of ketonic steroid sulphates might form dipolar ions - cf. some peculiarities of aminophenyl and aminonaphthyl sulphates, Burkhardt and Wood (1929)).

(7) General study of the conjugates (as such) throughout pregnancy.

The results of all the above work would pave the way for a systematic study of the conjugates in mare's pregnancy urine (and other horse urines) as such. The methods would be developed in the light of the results of (4), (5) and (6); the work should be at least on the scale indicated for item (4).

(8) Application of the Work in Veterinary Medicine.

Just as abnormalities in the nature and quantity of urinary steroid constituents may be of considerable diagnostic significance in human medicine, the same may be true in the veterinary world. Work on pathological horse urines cannot be started until some knowledge of normal urines has been gained.

The above suggestions are extensive, but they represent the minimum necessary for an adequate study of/

of the conjugated sulphates of mare's pregnancy urine. A very rough calculation suggests that the problems listed here might be attacked with some prospect of success on the following scale:-

Staff - 4 Graduates
2 Laboratory assistants
1 Stable man

Stud - 6 Mares

Time - 5 Years

Appendix B.

Page

Literature on the Steroids of Horse Urine

Non-carbinol/

Page

Non-carbinol Ketones

Steroids of Other Horse Urines

1. Introduction

Much scattered work has been done on the steroids of horse urine (principally pregnant mare's urine). About 100 papers (marked with an X in the list of references in Appendix D) have been traced.

Apart from the few papers on steroid sulphates mentioned in Part 1 of this thesis, all the work has been done on the free steroids present after hydrolysis of the urine. Much work has been directed to isolating oestrone for use in other fields. The only long series of papers is that by Marker and his co-workers at Pennsylvania State College. These workers, like some others, have been provided by commercial firms with residues remaining after the extraction of oestrone from very large volumes of urine. The treatment to which the products have been subjected in the factory is not disclosed in such work. Further, Marker subjects his crude material to such harsh treatment in the laboratory (e.g. steam distillation with 20% aqueous sodium hydroxide) that many of his compounds may be artefacts.

The literature on pregnant mare's urine is considered first (pp. 136 - 154) followed by that on non-pregnant mare's, stallion's and gelding's urine (pp. 153, 155-158)

Nearly/

Nearly 30 steroids have been isolated from pregnant mare's urine. A useful check on the author's own literature search was provided by the bibliography given by Prelog and Führer (1945).

Two tables are included giving the properties of the compounds isolated from pregnant mare's urine[x] and any data available on the quantities present. (These are sometimes estimates of quantities in the urine from biological or colorimetric determination ; sometimes statements of quantities isolated, which represent an unknown fraction of the quantity originally present). The only steroid whose excretion at various stages of pregnancy has been studied with any degree of thoroughness is oestrone.

STEROIDS OF PREGNANT MARE'S URINE

Phenolic Steroids

See Table 18, p.146.

Ketonic Phenols

2. Oestrone - Identification; Extraction; Hydrolysis of Conjugates.

Zondek(1930, 1931) first showed that PMU contained oestrogenic material ('combined folliculin?') which was not precipitable by ethyl alcohol and not/

x The abbreviation 'PMU' is used for 'pregnant mare's urine'.

not extracted by lipoid solvents. The oestrogenic material could be made ether-soluble by treatment with acid. PMU contained much more oestrogenic material than did human pregnancy urine.

de Jongh, Kober and Laqueur (1931) proved the identity of the oestrogen obtained from PMU by hydrolysis with the 'menformone' (oestrone) of human pregnancy urine.[x]

Stammler and Djatlowa (1932), and Lipschütz and Poch (1932) showed independently that the 'folliculin' of PMU could be transformed into an ether-soluble form by acid hydrolysis.

Butenandt and Störmer (1932) repeated the work of de Jongh et al. and claimed that they obtained from PMU a 'β-follicular hormone' different from the 'α-follicular hormone' of human pregnancy urine. Subsequently Butenandt and Jacobi (1933) stated that de Jongh et al. and Girard et al. (1932,a) were correct in claiming that the α-hormone was the principal one in PMU. de Jongh et al. (1934) finally showed that the supposed α- and β-hormones were the same substance (now called oestrone).

Curtis/

x See also Nieuwenkamp and Kober (1931), Dingemanse et al. (1931) on crystal structure and absorption spectra.

Curtis (1933) and Ito and Hayazu (1933) described methods for the extraction of oestrone from PMU after acid hydrolysis by adsorption on benzoic acid and charcoal respectively.

Extraction methods involving the formation of esters with phthalic anhydride, salicyl chloride, etc. by the oestrone-containing fractions were patented by Schering-Kahlbaum AG.(1933).

Beall and Marrian (1934) developed an extraction method using acid hydrolysis, toluene extraction separation of a 'weak phenolic' fraction (by distribution between different organic solvents and different concentrations of aqueous alkali), precipitation of oestrone as a mercury-ammonia complex and decomposition of this with acid.

Schachter and Marrian (1936) showed that the bulk of the oestrogenic material in PMU was present as ether-insoluble conjugates, and that there was no rise in the proportion of ether-soluble material immediately before delivery (as in the human species).[x] These authors (1938) isolated oestrone sulphate from PMU (see part I of this thesis, which also gives details of work on the same subject by Butenandt and Hofstetter (1939)).

Van/

x see also Marrian (1937).

Van Stolk et al. (1936, 1937) describe the industrial preparation of oestrone from PMU and its spectro-photometric estimation.

Edson and Heard (1939) found that the best conditions for the hydrolysis of the conjugated oestrogens of PMU were standing at room temperature for 4 weeks at pH 0.4-0.6.

3. Oestrone excretion at different stages of pregnancy

The analytical methods used have not really determined oestrone, but either total oestrogenic material (Allen-Doisy test) or chromogenic material (Kober's phenol-sulphuric acid colorimetric method (Kober, 1931; Cohen and Marrian, 1934)).

The most thorough study is by Kober (1935 a, b; 1938) using his own colorimetric method. In his 1938 paper Kober studied the excretion of oestrogens by 31 mares from the 6th month of pregnancy to term. The greatest excretion was during the 7th, 8th and 9th months (see Table 19).

Table 19 /

Table 19.

Excretion of chromogenic oestrogen ('oestrone') in PMU. Kober (1938).

Month	mg. oestrogen per litre	
	Mean	S.D.
6	17.7	10.0
7	23.9	7.8
8	24.0	9.2
9	22.7	8.5
10	18.1	9.0
11	8.8	4.0
12	5.9	3.7

Kober pointed out that the quantities vary greatly from mare to mare and from week to week in the same mare.

Most other work (done on smaller numbers of animals) supports Kober's work. Glud et al. (1933) showed a rise in oestrogen excretion from 2nd to 5th months. Hart and Cole (1934) showed a steady fall in oestrogen excretion during the last three months. Cole and Saunders (1935) studied 3 mares carefully and showed maximum excretion at the 7th month. The only discordant results are those of Küst (1934) who claimed a large increase in oestrogen excretion towards the end of pregnancy. In view of the weight of evidence against Küst, this work must be discredited.

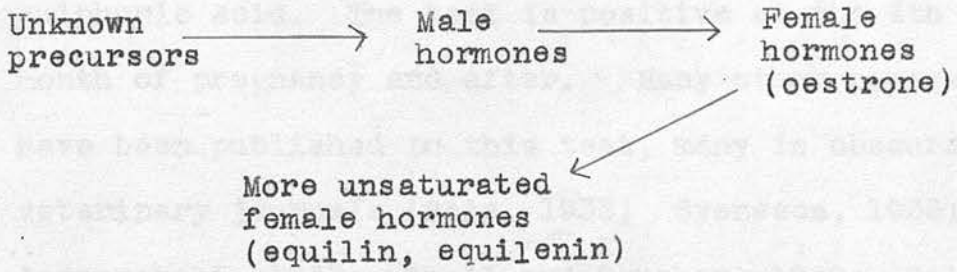
Beall/

Beall (with Edson, 1936) followed one mare throughout pregnancy and found a maximum (much greater than Kober's) of 100 mg. per litre at the 7th and 8th months. This figure was obtained by colorimetric determinations on the weak phenolic fraction. Edson and Heard (1939), after developing better methods of hydrolysis, confirmed this figure. (In some cases the maximum was as high as 150-200 mg. per litre).

4. Oestrone - miscellaneous work of biological character.

Origin and Function of Oestrone.

Glud et al. (1933) discussed the reasons for the late appearance of oestrone in PMU. Cole et al. (1933), Catchpole and Cole (1937), Hart and Cole (1937), considered the origin of the oestrone and finally suggested that it was formed in the placenta. Zondek (1934,b) discussed the metabolism of the sex hormones and suggested the following scheme:



The large quantities of oestrone in stallion's urine (see later) are supposed to be degradation products/

products from excess male hormone produced in the testes.

Campbell and Hey (1944) noted that the oestrone content of different urines (human, pregnant mares, stallion's) is approximately proportional to their p-cresol content. They suggested that some of the urinary p-cresol may be formed by degradation of oestrogens. Experimental investigation of this problem is necessary; attempts to isolate possible dicyclic and tricyclic intermediates would be worth while.

The metabolism of the oestrogens has recently been reviewed by Marrian (1946).

Pregnancy Diagnosis

Küst (1934) and Richter and Gehring (1935) used the Allen Doisy test on PMU for pregnancy diagnosis.

Cuboni (1934, 1937, 1938) developed a colorimetric method for pregnancy diagnosis which depends on the formation of a green fluorescence when a benzene extract of PMU is treated with concentrated sulphuric acid. The test is positive at the 4th month of pregnancy and after. Many other papers have been published on this test, many in obscure veterinary journals (Sala, 1935; Svensson, 1936; Jastrzebski, 1938; Sholl and Dersham, 1939; Cole and Hart, 1942, Olbrycht, 1942). Day and Miller (1940) compared this test with other methods for pregnancy diagnosis; of all tests, the mucin test, which can be/

be applied in the 3rd month, is the best.

Kober (1940) applied his naphthol sulphuric acid reaction (Kober, 1932,a) to pregnancy diagnosis.

Clinical Uses.

Six papers have been published on the clinical use of a water-soluble oestrogen preparation from PMU ('Premarin' - claimed to be essentially sodium oestrone sulphate) - Goodall (1942); Freed, Elsin and Greenhill (1943); Gray (1943); Glass and Rosenblum (1943); Serringhaus and St John (1943); Turner, Davis and Hamblen (1943).

5. Equlin, Hippulin and Equilenin

These three ketonic phenols were obtained together with oestrone by Girard and his co-workers (Girard et al. 1932, a,b,c; Sandulesco et al., 1933; Girard, 1933). The three new ketones are more unsaturated than oestrone, equilin (X) and hippulin (XI) having one more double bond, and equilenin two more.[x] Equilenin (XII) contains a naphthalene nucleus and Sandulesco et al. (1933) describe the separation of this compound as its sparingly soluble picrate.

Girard (1933) mentions the use of the then new quaternary-ammonium-salt-hydrazine reagents of Girard and Sandulesco (1936) in the separation of these compounds.

Little/

x Structural formulae (X et seq.) are on pp. 172-174

Little subsequent work has been done on these compounds. Beall (with Edson, 1936) while improving the oestrone isolation method of Beall and Marrian (1934) obtained equilin from the fraction not precipitated by mercury.

Girard (1933) stated that early and middle PMU contained little or no equilin or equilenin, and that the excretion of these compounds increased greatly towards the end of pregnancy.[x] This was confirmed by Edson and Heard (1933).

Duschinsky and Lederer (1935) describe the separation of oestrone and equilenin by chromatography.

3-Desoxyequilenin has been isolated from PMU by Prelog and Führer (1945)(see p. 152 of this thesis). See also Marker et al., 1939,c.

Non-ketonic Phenols

6. 'δ-Follicular Hormone', α- and β-Oestradiols, 17-Dihydroequilenin

Schwenk and Hildebrandt (1932) obtained from PMU a new hormone $C_{18}H_{22}O_2$ (m.p. 209°) which had a higher activity in the Allen-Doisy test than any previously studied compound. (They pointed out that de Jongh et al. (1931) and Butenandt and Störmer (1932) had obtained small quantities of a substance of similar physical properties). They suggested that their compound $C_{18}H_{22}O_2$ which they called 'δ-follicular hormone' might be a new hormone or a molecular compound of one of the known hormones with/

x See also Cartland and Meyer, 1935.

with some unknown material.

Wintersteiner, Schwenk and Whitman (1935), again obtained from PMU ' δ-follicular hormone, which they showed to be a diol, together with α-oestradiol (XIII) and a third substance of m.p. 236° (uncorr.).

' δ-Follicular hormone' was investigated further by Wintersteiner et al. (1936), Wintersteiner (1937), Hirschmann and Wintersteiner (1938,a), who finally showed that it was a 1:1 molecular compound of β-oestradiol (XIV) with 17-dihydro-equilenin(XV).

Hirschmann and Wintersteiner (1938, a, b) also obtained from PMU very small quantities of an unsaturated diol ('Compound 3', m.p. 220-223.5°) which they suggested might be Δ^6-17 dihydro-equilin.

Sandulesco and Laboratoires Françaises de Chimiotherapie (1936) patented a process for obtaining oestradiol from PMU. The non-ketonic extract was treated with benzoyl chloride in aqueous alkali at room temperature to benzoylate phenolic hydroxyl groups. The alcoholic groups were then esterified with chloracetic acid and the halogenated esters condensed with tertiary bases to give water-soluble quaternary ammonium salts. These on saponification yielded oestradiol.

Van /

Table 18

ENOLIC STEROIDS OF PREGNANT MARE'S URINE.

Molecular Formula	M.p.	Specific Rotation	Derivatives (with m.p.s)	Quantity in urine Estimated mg./l.	Isolated mg./l.
$C_{19}H_{22}O_2$	259	+170D	acetate, 126 semicarb.266-7c.	110(max)	35 (max)
$C_{18}H_{20}O_2$	238-240 c.	+308D	benzoate, 198c. semicarb., 265-7c.		0.15
$C_{18}H_{20}O_2$	233c.	+128D			v.small
$C_{18}H_{18}O_2$	259c.	+87D	acetate, 157 semicarb., 268		0.03
$C_{18}H_{24}O_2$	176-8c.	+81A	diacetate, 127c.		1.3
$C_{18}H_{24}O_2$	220-3c.	+54D	diacetate, 139-141c.		0.003
$C_{18}H_{20}O_2$	215-7c.	-5D	diacetate, 115-7c.		0.015
$C_{18}H_{22}O_2$	220-4				v.small

ormulae on p. 172 which folds

t room temperature in the
: dioxan.
ax. = maximum.

the hydroxy compound present in PMU, from which their 3-desoxy-11-keto equilenin had been obtained, might be 3-desoxy-17-dihydro-equilenin (oestra-pentene-17-ol)(XVa).

Non-ketonic Carbinols.

8. C-21 Diols.

The following have been obtained from PMU by Marker et al.

Pregnane-3(α),20(α)-diol (XVI), 1937.
allo-Pregnane-3(α),20(α)-diol (XVII), 1938,j.
allo-Pregnane-3(β),20(α)-diol (XVIII), 1938,g.
Pregn-5-ene-3(β),20(α)-diol (XIX), 1938,g.
Urane-3(β),11-diol (XX?) , 1938,f.

XVI has also been obtained from PMU by Weil (1938).

Marker et al. (1938,j) gave figures (see below) for the approximate quantities of the pregnane and allo-pregnane diols isolated from PMU which were taken as support for the generalisation that the proportions of these three diols were the same in a number of different urines. Subsequently (1939,b) these authors gave totally different figures, stating: 'The figures (previously) reported for mare's pregnancy urine were arbitrarily(!) based upon the amount of allo-pregnanedione obtained from the oxidation of the total carbinol fraction .... Recently we have obtained indications that mare's pregnancy urine differs considerably from the other urines studied, especially in regard to the ratio of pregnanediols present.'

mg./

	mg./gallon urine. approx. quantities isolated (1938,j)	approx. amounts indicated (1939,b)
Pregnane-3(α),20(α)-diol	50	3
allo-Pregnane-3(α),20(α)-diol	25[x]	2
allo-Pregnane-3(β),20(α)-diol	6	25

x I have been unable to find any details of the isolation of this compound.

Pincus and Pearlman (1943, at p. 301) appear to have overlooked Marker's change of opinion regarding the concentration of PMU diols expressed in his 1939 paper.

Marker's suggested structure for uranediol (XX) has been criticised on pp. 106-110 of this thesis. The present author's Compound Y (Part 4 of this thesis) is certainly a saturated diol, and may be identical with Marker's uranediol (see pp. 105-106).

The C-21 diols, hydroxy ketones and diketones with substituents at positions 3 and 20 are all possible reduction products of progesterone - see review by Pincus and Pearlman (1943).

9. C-21 Triols.

Two triols have been isolated, the first(m.p. 300-302°) by Smith, Hughes, Marrian and Haslewood(1933), and Haslewood, Marrian and Smith (1934). This compound was subsequently isolated by Marker et al.(1938) and called/

called pregnanetriol B. Its constitution has been the subject of much discussion. Odell and Marrian (1938) suggested that it was a 3,6,20 triol; Marker et al. (1938,c) claimed that it was a 3,4,20 triol. After further work the latter authors changed their opinion (1939,a) and suggested that it was allo-pregnane-3(α),16,20-triol (XXI). They subsequently (1940,a) produced further evidence in favour of this structure, the triol being oxidised by Oppenauer's method to allo-pregn-16-ene-3,20-dione.

The other C-21 triol (m.p. 295-300°) was isolated by Marker et al. (1938,a) and called 'Pregnanetriol A'. These authors subsequently, on insufficient evidence, assigned to this compound the structure of a uranetriol (XXII).

10. Equistanol

Marker et al. (1938,g) isolated from PMU a mono-hydroxy compound previously obtained (same authors, 1938,d) from stallion's urine. This compound, which gave analytical figures corresponding to $C_{30}H_{54}O$ or $C_{31}H_{56}O$, could be oxidised to a monoketone. It was precipitated by digitonin and was named by Marker β-equistanol.

No further work on this compound appears to have been done and its steroid character has not been proved.

Ketonic/

Ketonic Carbinols

11. C-18 Compound

Heard and Hofmann (1940, 1941) obtained a compound $C_{18}H_{22}O_2$ which could be reduced catalytically to a known oestrane-3-17-diol and in which the B-ring was aromatic. They suggested for this compound the structure XXIII, oestra-5,7,9-triene-3(β)-ol-17-one.

12. C-19 Compounds

Dehydro-iso-androsterone (XXIV) has been isolated from PMU by Oppenauer (1941[x]). The same author claims the isolation of two hydroxy-ketones, C ($C_{19}H_{30}O_2$, precipitated by digitonin, no other details given) and D ($C_{18-19}H_{22}O_2$, not precipitated by digitonin). D is said to contain three non-conjugated olefinic linkages.

An androstanolone isomeric with androsterone was isolated by Heard and McKay (1939, 1941) and subsequently by Oppenauer (1941). Heard and McKay (1939) oxidised the hydroxy-ketone to a diketone which was reduced (Clemmensen) to androstane; the hydroxy-ketone was precipitable by digitonin. These authors/

x The original paper has not been available for consultation - an unsatisfactory abstract in Chemical Abstracts had to be used.

authors suggest (1941) that the keto group is at C-15, by exclusion of all other positions. The formula XXV is therefore proposed for this compound.

13. C-21 Compounds

Marker et al. (1938,e) and Heard and McKay (1939) have isolated from PMU allo-pregnane-3(β)-ol-20-one (XXVI). The present author's compound Z (see Part 3 of this thesis and Klyne and Marrian, 1945) is probably allo-pregn-16-ene-3(β)-ol-20-one (XXVII), but further evidence on its constitution is necessary.

Three other hydroxy ketones ($C_{21}H_{34}O_2$) of unknown structure have been obtained from PMU, viz. 'uranolone' (Marker et al, 1938,e; structure XXVIII suggested by author); Compounds A and B (Oppenauer, 1941; these are both precipitated by digitonin).

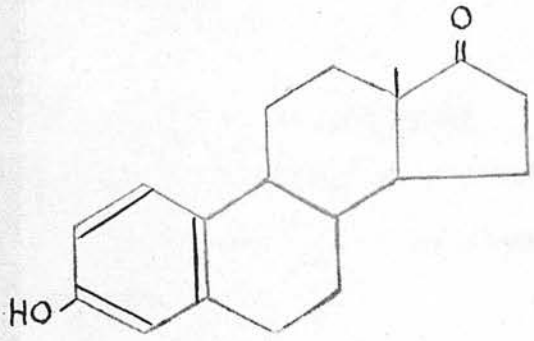
Non-carbinol Ketones

14. C-18 Compound.

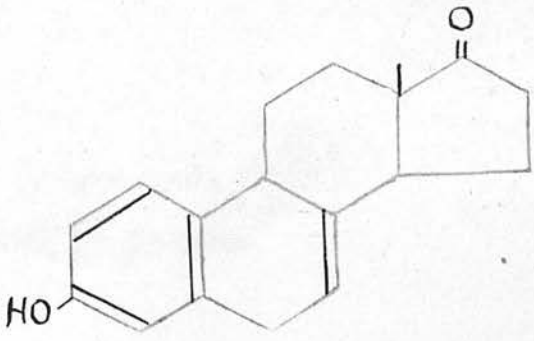
Prelog and Führer (1945) isolated from PMU a mono-ketone $C_{18}H_{18}O$ which they reduced catalytically to the known 3-desoxy-hexahydro-equilenin(XXIX). The absorption spectrum of the original ketone closely resembled that of 1;2:3:4-tetrahydrophenanthrene, and the authors suggest for their compound the structure XXX (3-desoxy-equilenin).

15 /

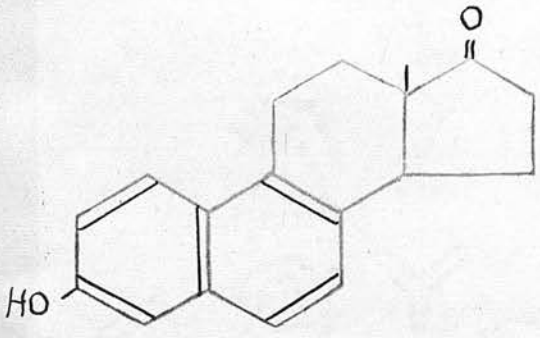
FORMULAE IX-XVI.



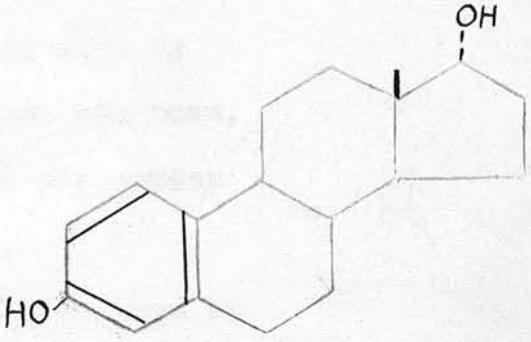
IX
Oestrone



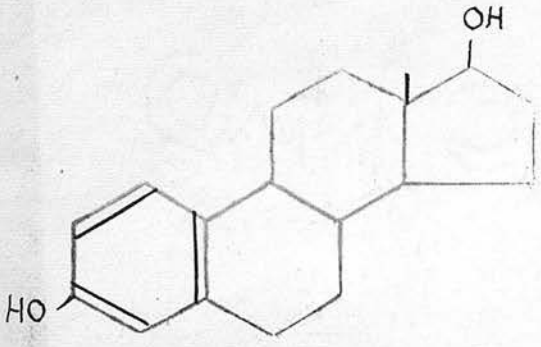
X
Equilin



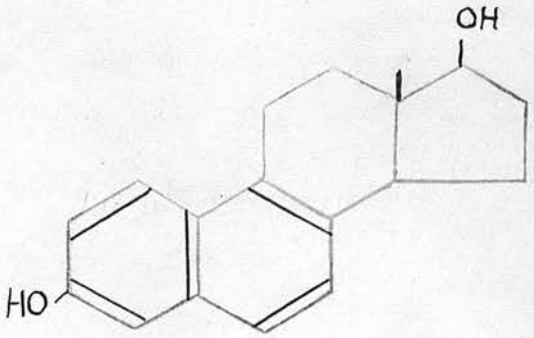
XII
Equilenin



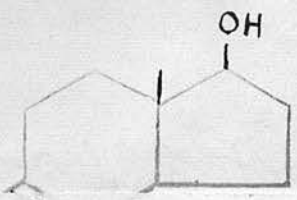
XIII
α-Oestradiol

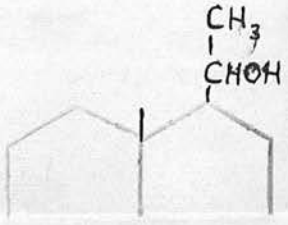


XIV
β-Oestradiol



XV
17-Dihydroequilenin





15. C-21 Compounds

Pregnane-3,20-dione (XXXI) and allo-pregnane-3, 20-dione (XXXII) have been isolated from PMU by Marker et al. (1938,e).

16. Keto-lactones

Heard (1938) obtained from PMU a compound $C_{19}H_{26}O_3 \pm CH_2$ (m.p. 252°, uncorr.), which gave a mono-semicarbazone. Subsequent unpublished work by Heard and Hofmann (referred to by Pincus and Pearlman, 1943, at p. 315) showed that the remaining two oxygen atoms were in the form of a lactone group.

An apparently identical compound was obtained by Jacobs and Laqueur (1939); these authors transformed their compound into a keto-hydroxy acid $C_{19}H_{28}O_4$ (m.p. 240-243°) by boiling with alcoholic potassium hydroxide. Market et al. (1938,e) obtained a substance which was probably this keto-lactone; their data are criticised by Jacobs and Laqueur.

It is tempting to suggest that this keto-lactone arises by oxidation of a diketone in a manner similar to that in which the lactone (XXXIII) is formed from oestrone (Westerfeld, 1942).

STEROIDS OF OTHER HORSE URINES

17. Non-pregnant Mare's Urine

Little work has been done on the steroid content of non-pregnant mare's urine.

Glud et al. (1933), Kust (1934), and Zondek(1934a,c) all/

Table 20

NON-PHENOLIC STEROIDS OF MARE'S PREGNANCY URINE

Compound	Molecular Formula	M.p.	Specific rotation	Derivatives (with m.ps)	Quantity in urine Estimated mg./l.	Quantity in urine Isolated mg./l.
NON-KETONIC CARBINOLS						
3-Dexoxy-17-dihydro-equilenin } (XVa)? Oestrapentene-17-ol }	$C_{18}H_{20}O$					NOT isolated
Pregnane-3(α),20(α)-diol (XVI)	$C_{21}H_{36}O_2$	243-4c.		diacetate, 168+183c $[\alpha]_D$ +26B	11, 0.7, ?	
allo-Pregnane-3(α),20(α)-diol (XVII)	"	248c.		" 142c $[\alpha]_D$ +19B	5, 0.4 ?	
allo-Pregnane-3(β),20(α)-diol (XVIII)	"	220-2c.		diacetate, 165-8c	1.3, 5 ?	
Pregn-5-ene-3(β),20(α)-diol (XIX)	$C_{21}H_{34}O_2$	176		" , 144-6		
Urane-3(β),11-diol (XX?) Klyne's Y	$C_{21}H_{36}O_2$	210 211-3c.	+2A	" , 160 " , 160 $[\alpha]_D$ -32Chf	1.4 5	1.4 3
Pregnanetriol B allo-Pregnane-3(α),16,20-triol } (XXI?)	$C_{21}H_{36}O_3$	303-5	-41Py (5461)	triacetate, 168		1.3
Pregnanetriol A Urane-3(α),11,20-triol } (XXII?)	$C_{21}H_{36}O_3$	295-300		" 136		2
β-Equistanol	$C_{30}H_{54}O$?	134		acetate, 124 ketone 115		
KETONIC CARBINOLS						
Oestra-5,7,9-triene-3(β)-ol-17-one (XXIII)	$C_{18}H_{22}O_2$	139	+59A	acetate, 158u oxime, 195-7d,u.		0.004
Dehydro-iso-androsterone(XXIV) } Androst-5-ene-3(β)-ol-17-one }	$C_{19}H_{28}O_2$	141c or 153c.	+11A	acetate, 172c		
Androstane-3(β)-ol-15-one(XXV) } Heard's compound ? }	$C_{19}H_{30}O_2$	187	-160D	benzoate, 206-8		
Oppenauer's A	$C_{21}H_{34}O_2$	163		acetate, 144; $[\alpha]_D$ +92A semicarb. 275-8		

Androstane-3(β)-ol-15-one(XXV) Heard's compound ?	$C_{19}H_{30}O_2$	187		-160D	benzoate, 206-8
Oppenauer's A	$C_{21}H_{34}O_2$	163			acetate, 144; $[\alpha]_D$ +92A semicarb. 275-8
" B			"	197	+93A	benzoate, 208 semicarb., 258-61
" C	$C_{19}H_{30}O_2$				
" D	$C_{18-19}H_{22}O_2$				acetate, 167-9; $[\alpha]_D$ +40A
allo-Pregnane-3(β)-ol-20-one (XXVI)	$C_{21}H_{34}O_2$	195-8c		+93Chf	acetate, 150-2c. $[\alpha]_D$ +74 Chf oxime, 225u.
Klyne's Z	$C_{21}H_{32}O_2$	205-7c		+50A	acetate 163-5c.	20 max
allo-Pregn-16-ene-3(β)-ol-20-one ? (XXVII)					
Urane-11-ol-3-one ? (XXVIII)	$C_{21}H_{34}O_2$	165			semicarb. 250d.
NON-CARBINOL KETONES					
3-Desoxy-equilenin (XXX) (Oestrapentene-17-one)	$C_{18}H_{18}O$	155-7		+117Chf		v.small
Pregnane-3,20-dione (XXXI)	$C_{21}H_{32}O_2$	118			disemicarb., 257d
allo-Pregnane-3,20-dione (XXXII)	$C_{21}H_{32}O_2$	203-5c.		+127A	" , 325
KETO-LACTONE					
Compound $C_{19}H_{26}O_3$ of Heard and Jacobs and Laqueur	$C_{19}H_{26}O_3$	252, 258			semicarb., ca 310d hydroxy-keto-acid, 240-3

Roman numerals indicate structural formulae on pp.172-174, which folds out clear of the thesis.

References are given in the text.

Specific rotations are for D light (except where marked '5461').
A = EtOH; Chf = chloroform; D = dioxan; B = benzene; Py= pyridine;
c = corrected; d = decomposes; u = uncorrected; max = maximum

Since the oestrogen concentration of PMU varies greatly from month to month, the figures have no precise significance; it appears, however, that the concentration in stallion's urine is of the same order as that in PMU, and far greater than that in non-pregnant mare's urine or gelding's urine.

Zondek (1934,a,c) showed that stallion's testes contained a higher concentration of oestrogen than any other organ (54,000 mouse units per kg.). He suggested (1934,b) the following path for the metabolism of the hormones:

Unknown precursor → Male hormones → Female hormones

and considered that the oestrogen in stallion's urine represented the excess of male hormone elaborated in the testes which was not required for use.

Cartland et al. (1935) showed that crystalline oestrone equivalent to 60% of the oestrogenic activity of stallion's urine could be obtained by acid hydrolysis, extraction with butyl alcohol and partitions between various solvents.

Wintersteiner et al. (1935) suggested that Zondek's high figures for oestrogen content might be due to presence of highly active diols. This suggestion was substantiated by Levin (1945) who isolated α-oestradiol from stallion's urine (5 mg. per litre). Levin stated that this oestradiol was responsible for 40-90% of/

of the total oestrogenic activity of the urine. Beall(1940) had isolated α-oestradiol (0.21 mg./kg.) and oestrone (0.36 mg./kg.) from stallion's testes.

Einhorn and Knorozova (1939) claimed that when a benzene extract of hydrolysed stallion's urine was extracted with 75% sulphuric acid, androgens passed into the latter. No details are given in the Abstract of this paper.

Marker et al. (1938,d) claim to have demonstrated the presence in stallion's urine of the following carbinols:

1. β-Equistanol; $C_{30}H_{53}OH$ or $C_{31}H_{55}OH$, m.p. 134°; gave a mono-acetyl derivative and on oxidation a mono-ketone; thought possibly a phytosterol.
2. α-Equistanol; not isolated; presence detected by epimerisation to the β-isomer by sodium in boiling zylene.
3. Allo-pregnane-triol; not isolated; presence detected as for 2 above; β isomer has m.p. 295° and gives a triacetate, m.p. 140-145°.
4. Allo-pregnanetetrol; not isolated; presence detected as for 2 and 3 above; β-isomer has m.p. 295°.
5. Uranetriol; not isolated; presence detected by oxidation to uranetrione.

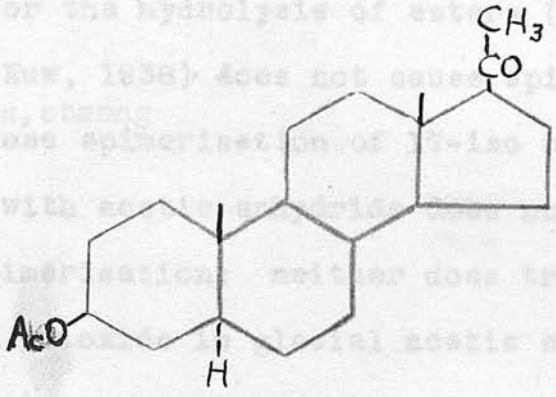
19. Gelding's Urine

The only references appear to be by Zondek (1934,a,c) who showed that the oestrogen content of gelding's urine was very low (400 mouse units per litre; about 0.3% of that of stallion's urine) and by Rea (1940) who claimed that extracts of gelding's urine had no effect on the reproductive organs of the male rat.

Appendix C.

SOME NOTES ON 17-ISO PREGNANE COMPOUNDS.

Butenandt and Fleischer (1935) obtained by the alkaline hydrolysis of allo-pregnane-3(β)-ol-20-one acetate (XXXIV) the expected hydroxy-ketone, and an isomer which they suggested was the C-17 epimeric compound (17-iso-allopregnane-3(β)-ol-20-one).



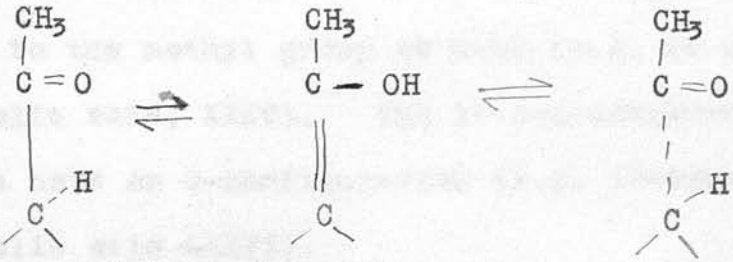
XXXIV.
allo-Pregnane-3(β)-ol-20-one acetate.

Table 21 (page 162) summarises the data available about 17-iso-pregnane and 17-iso-allo-pregnane compounds, in which a hydrogen atom is attached to C-17. Fifteen such compounds have been traced, and in all of these C-20 carries a carbonyl oxygen

Epimerisation/

Epimerisation at C-17.

The ready interconversion of 17-n and 17-iso compounds by heating with alkali is presumably due to the enolisation of the 20-keto group as follows.



It may be noted that the mild potassium bicarbonate method for the hydrolysis of esters (Reichstein and von Euw, 1938) does not cause epimerisation at C-17. In many cases, strong
/acids cause epimerisation of 17-iso compounds. Heating with acetic anhydride does not appear to cause epimerisation; neither does treatment with chromium trioxide in glacial acetic acid (with one exception).

Specific Rotations

In all known cases, the 17-iso-compound is more laevo-rotatory than the 17-n-compound.

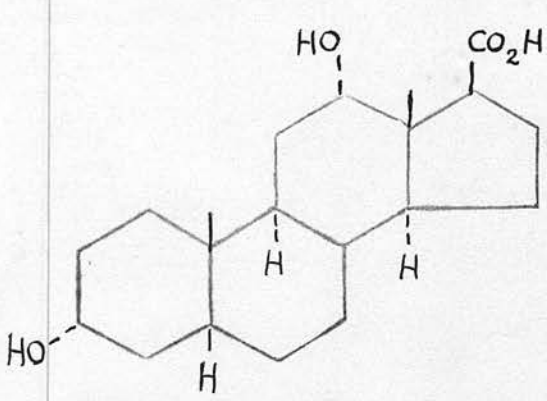
Melting-Points

In most, but not all cases, the 17-iso-compound melts lower than the 17-n-compound.

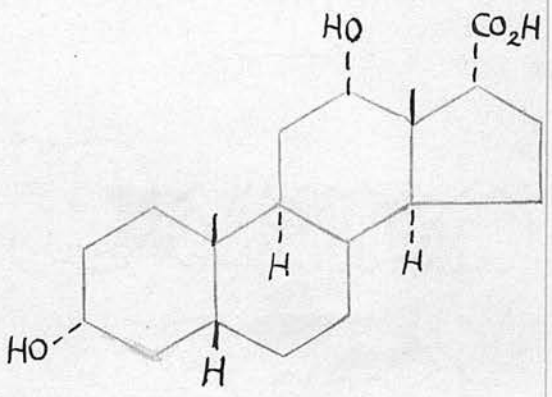
Configuration/

Configuration at C-17.

A recent paper by Sorkin and Reichstein (1946) on the aetiodesoxycholic acids makes it all but certain that the side chain in all naturally-occurring steroids and their derivatives occupies the β-position i.e. cis to the methyl group at C-10 (e.g. in aetiodesoxycholic acid, XXXV). The 17-iso-compounds must therefore have an α-configuration (e.g. 17-iso-aetiodesoxycholic acid XXXVI).



XXXV.
Aetiodesoxycholic acid



XXXVI.
17-iso-Aetiodesoxycholic acid

This paper gives references to previous chemical and crystallographic work on the configuration at C-17 and states that papers on the configuration of C-17 hydroxy-steroids will be published shortly.

Table 21

17-ISO-PREGNANE AND 17-ISO-ALLO-PREGNANE COMPOUNDS

Compound	17-normal m.p.	17-normal $[\alpha]_D$	17-iso m.p.	17-iso $[\alpha]_D$	Interconversion of 17-normal and 17-iso Compounds	References
AA allo-Pregnane-3(β)-ol-20-one	194	+91A	148	+6A	b.5% MeOH-KOH gives equilibrium mixture Ac_2O does not epimerise either compound iso-AA with CrO_3/HOAc gives iso-FF and some n-FF	1
BB Acetate of AA	144	101	101		n-BB or iso-BB on hydrolysis with b. MeOH-KOH gives mixture of n-AA and iso-AA	1
CC Pregn-5-ene-3(β)-ol-20-one	190	+28A	173	-141A	b.5% MeOH-KOH gives equilibrium mixture Ae_2O does not epimerise either compound iso-CC on Oppenauer oxidation gives iso-EE	2,3
DD Acetate of CC	147	+20A	171	-126A	As for BB. iso-DD is hydrolysed by b. $KHCO_3$-MeOH-aq. to iso-CC without much epimerisation	2,3
EE Pregn-4-ene-3,20-dione (Prpgesterone)	128	+193A	145	0 A	iso-EE with b.HCl-EtOH-aq. gives EE	3
FF allo-Pregnane-3,20-dione	200	+127A	135? 149?	-15A	KOH-MeOH or NaOMe-MeOH gives equilibrium mixture Ac_2O does not epimerise iso-FF.	1,4
GG 3(β),21-Diacetoxy-allo-pregnane-11-ol-20-one (R diacetate)	174	+84D	133 and 148	-60An	iso-GG not epimerised by b.AcOH or b.HCl/AcOH or CrO_3/AcOH iso-GG with b. conc.aq.HCl is epimerised (and loses 11-OH)	5
HH 3(β),21-Diacetoxy-allo-pregnane-11,20-dione (N diacetate)	149	+78An	132	-44An	iso-HH not epimerised by b.pyridine or b.$MeNO_2$ iso-HH with b.aq.alc.HCl gives HH	5
JJ Pregn-4-ene-21-ol-3,20-dione (Desoxycorticosterone)	142c.	+178A	181	-6A		
KK Acetate of JJ	161	+177A	137 and 174	-26An	iso-KK with b.$KHCO_3$-MeOH-aq. gives iso JJ; with b. aq. alc. HCl gives KK	6
LL Pregnane-3(α)-ol-20-one	151	+112Me	144	-41D	2.5% NaOH-MeOH or 4.3% HCl-MeOH gives equilibrium mixture	7
MM Acetate of LL	99	+123	159	-28Me		7
NN Oxime of LL	224-6		192-7			7
PP Pregnane-3(α),12(α)-diol-20-one	192	+8 Chf	233	-46 Chf	K_2CO_3-MeOH-aq. gives equilibrium mixture iso-PP with CrO_3/HOAc gives iso-QQ	8
QQ Pregnane-3,12,20-trione	204-6	+167An	153	+58An	KOH-MeOH gives equilibrium mixture	8

References

1. Butenandt and Mamoli (1935)
2. Butenandt and Fleischer (1937)
3. Butenandt, Schmidt-Thome and Paul (1939)
4. Marker et al.(1939,e)
5. Shoppee and Reichstein (1940)

b. = boiling
(A = ethyl alcohol

Specific

Appendix D

REFERENCES

These are given as in the Biochemical Journal.

References to work on horse urines are marked with a x.

Allen,C.F.H. and Blatt,A.H.(1943). Article on 'Unsaturation and Conjugation' in Gilman 'Organic Chemistry', 2nd ed. Wiley, New York, at p. 678.

x Anderson, J. (1941). Vet. Rec. 53, 251.

Barnett,Jean, Henly, A.A. and Morris,C.J.O.R.(1946). Biochem. J. 40, 445.

Barton, A.D. and Young,L.(1943). J.Amer.Chem. Soc. 65, 294

x Baumann, E.(1876a). Ber,dtsch.chem.Ges. 9, 55.
x --- (1876,b). Ber.dtsch.chem.Ges. 9, 1389.
x --- (1878-9). Hoppe Seyl.Z. 2, 335.

x Beall,D.(1940). Biochem.J. 34, 1293.

x Beall,D.,with Edson,M. (1936). Biochem.J. 30,577.

x Beall,D. and Marrian, G.F.(1934). J.Soc.chem.Ind.53,309T.

Burkhardt,G.N.and Wood,H.(1929). J.Chem.Soc. 141.

Butenandt,A.and Fleischer,G.(1937).Ber.dtsch.chem. Ges. 70, 96.

Butenandt,A. and Hannsch,G.(1935). Hoppe Seyl.Z.237,96.

x Butenandt,A. and Hofstetter,H.(1939) Hoppe Seyl.Z.259,222.

x Butenandt,A. and Jacobi,H.(1933). Hoppe Seyl.Z.218,104.

Butenandt,A. and Mamoli,L.(1935). Ber.dtsch.chem.Ges.68,1847.

Butenandt,A., Mamoli,L. and Heusner,A.(1939). Ber.dtsch. chem.Ges. 72, 1617.

Butenandt,A., Schmidt-Thomé,J. and Paul,H.(1939). Ber. dtsch.chem.Ges. 72, 1112.

x Butenandt/

x Butenandt,A. and Störmer,I.(1932). Hoppe Seyl.Z.208,129.

Butenandt,A. and Westphal,U. (1936). Ber.dtsch.chem. Ges. 69, 447.
--- --- and Hohlweg,W.(1934). Hoppe-Seyl.Z.227,98.
Callow,N.H., Callow,R.K.,Emmens,C.W. and Stroud,S.W. (1939). J. Endocrinol. 1, 76.

x Campbell, N.R. and Hey,D.H.(1944). Nature, Lond.153,745.
x Cartland,G.F.and Meyer,R.K.(1935). J.biol.Chem.112,9.
x Cartland,C.F., Meyer,R.K.,Miller,L.C. and Rutz,M.H. (1935). J.biol. Chem. 109, 213.

x Catchpole,W.R. and Cole,H.H.(1934). Anat. Rec.59,335.

Cohen,S.L. and Marrian, G.F.(1934). Biochem.J.28,1603.

x Cole,H.H. and Hart,G.H.(1942). J.Amer.vet.med.Ass.101,124.

x Cole,H.H., Hart,G.H.,Lyons,W.R. and Catchpole,W.R. (1933). Anat. Rec. 56, 275.

x Cole,H.H. and Saunders, F.J.(1935). Endocrinology,19,199

x Cuboni,E. (1934). Klin.Wschr. 13, 302.

x --- (1937). Clin.Vet.Milano. 60,375.

x --- (1938). Boll. Soc.ital.Biol.sper. 13,433; Brit.Chem.Abstr/AIII,1101. /1938

x Curtis,J.M.(1933). J.biol.Chem. 100,xxxiii.

x Day,F.T.and Miller,W.C.(1940). Vet.Rec. 52,711.

x Deulofeu,V. and Ferrari,J.(1934,a). Nature,Lond.133,835.
x --- (1934,b). Hoppe-Seyl.Z.226,192.

x Duschinsky,R. and Lederer,E.(1935). Bull.Soc.Chim. Biol. 17,1534.

x Edson,M. and Heard,R.D.H.(1939). J.biol.Chem.130,579.

x Einhorn,G.L. and Knorozova,G.V.(1939). Biochimia,4, 575 (Brit. Chem. Abstr. 1941,AIII,676).

x von Euw,J. and Reichstein, T.(1942). Helv.Chim.Acta,25,988.

x Frattini,B.(1936). Boll.Soc.ital.Biol.sper. 11, 853. (Brit. Chem. Abstr. 1937, AIII,185).

x Freed/

x Freed, S.C., Elsin, W.M. and Greenhill, J.P.(1943).J. Clin. Endocrinol. 3, 89.

x Girard,A.(1933). Bull. Soc.Chim. Biol. Paris. 15,562.

Girard, A. and Sandulesco,G.(1936). Helv.chim.Acta,19,1095.

x Girard,A., Sandulesco,G., Fridenson,A. and Rutgers, J.J. (1932,a). C.R.Acad.Sci. Paris, 194, 909.

x Girard,A., Sandulesco,G., Fridenson,A.,Rutgers,J.J. and Gaudefroy, C. (1932,b). C.R.Acad.Sci. Paris, 194, 1020.

x Girard,A., Sandulesco,G., Fridenson,A. and Rutgers, J.J. (1932,c). C.R.Acad.Sci. Paris, 195, 981.

x Glass, S.J. and Rosenblum, G. (1943). J.clin. Endocrinol. 3, 95.

x Glud,P,Pedersen-Bjergaard,K. and Portman.K(1933). Endokrinologie, 13, 21 (abstract in Endocrinology, 18, 549)

x Goodall, J.R.(1942). J. Obstet. Gynaec. 49, 660.

x Gray, L.A. (1943). J.clin. Endocrinol. 3, 92.

Hanson, S.W.F.,Mills,G.T. and Williams,R.T.(1944). Biochem.J. 38, 274.

x Hart,G.H. and Cole,H.H.(1934). Amer.J. Physiol.109,320.

x Haselwood, G.A.D., Marrian, G.F. and Smith, E.R. (1934). Biochem.J. 28, 1316.

x Häussler, E.P.(1934). Helv. chim. Acta, 17, 531.

x Heard, R.D.H. (1938). J. Amer.chem.Soc. 60,493.

x Heard, R.D.H. and Hofmann, M.M.(1940). J.biol.Chem. 135, 801.

x Heard, R.D.H. and Hofmann,M.M.(1941). J.biol.Chem. 138, 651.

x Heard, R.D.H. and McKay,A.F.(1939). J.biol.Chem. 131, 371.

x Heard, R.D.H. and McKay,A.F.(1941). J.biol. Chem. 140, lvi.

x Hirschmann/

x Hirschmann,H. and Wintersteiner,O.(1938,a). J.biol. Chem. 122, 303.

x Hirschmann, H. and Wintersteiner,O.(1938,b). J.biol. Chem. 126, 737.

Hoehn, W.M. and Mason,H.L. (1938). J.Amer.chem.Soc.60,1493.

x Hofstetter,H.(1939). Dissertation, Berlin.

Hurd, C.D.(1938). Article on 'Open-chain Nitrogen Compounds' in Gilman,H. 'Organic Chemistry'. Wiley, New York. 1st ed. Vol.I at p. 629.

x Ito,M. and Hayazu,S.(1934). Munch.med.Wschr. 80,1969.

x Jacobs, J.D. and Laqueur,E.(1939). Rec.Trav.chim.58,77.

x Jastrzebski. (1938). Wet. Wspol. 5, 132.

x de Jongh, S.E., Kober,S. and Laqueur, E.(1931). Biochem.Z. 240, 247.

x de Jongh, S.E. Kober,S. and Laqueur,E. (1934). Biochem.Z. 270,17.

x ~~Kemp, T. and Pedersen-Bjergaard,K. (1933). Endokrinologie, 13, 156.~~

x Klyne, W. (1946). Biochem.J. 40, Proc. (in the press)

Klyne,W. and Bell, C.(1946). Biochem.J. 40,Proc. liv.

x Klyne,W. and Marrian, G.F. (1945). Biochem.J. 39, Proc. xlv.

Kober, S.(1931). Biochem.Z. 239, 209.

x --- (1935,a). Klin.Wschr. 14, 381.

x --- (1935,b). Acta Brev.neerl.Physiol.5, 34.

x --- (1938,a). Biochem.J. 32, 357.

x --- (1938,b). Arch. int.Pharmacodyn. 59, 61.

x --- (1940). Endocrinology, 26, 914.

x Küst (1934). Klin.Wschr. 13, 1782.

x Lederer/

x Lederer, E. (1943). Trav. Soc.Chim.biol. 25, 1237.

x Levin, L.(1945). J.biol.Chem. 158, 725.

x Lipschütz, A. and Poch,E.(1932). C.R.Soc. Biol. Paris. 111, 856.

The following papers by R.E.Marker and his school are all indicated in the text as 'Marker et al.' Marker's chief co-workers in this branch of steroid chemistry have been O.Kamm, E. Rohrmann and E.L.Wittle.

x Marker,R.E. et al.(1937). J.Amer.chem.Soc. 59, 2297.

x --- (1938,a) --- 60, 210.

x --- (1938,b) --- 60, 1061.

x --- (1938,c) --- 60,1067.

x --- (1938,d) --- 60,1555.

x --- (1938,e) --- 60,1559.

x --- (1938,f) --- 60,1561.

x --- (1938,g) --- 60,1565.

x --- (1938,h) --- 60,1567.

x --- (1938,j) --- 60,2931.

x --- (1939,a) --- 61,855.

x --- (1939,b) --- 61,2537.

x --- (1939,c) --- 61,3314.

x --- (1939,d) --- 61,2719.

x --- (1939,e) --- 61,1335.

x --- (1940,a) --- 62,2540.

x --- (1940,b) --- 62,3003.

Marker,R.E. et al.(1940,c). J.Amer.chem.Soc. 62,3350.

--- (1944). U.S. patent 2,345,711 (Chem. Abstr.(1944) 38, 4760.)

x Marrian, G.F.(1937). Cold Spr. Harb. Sym. quant. Biol. 5, 16.

x Marrian, G.F.(1946). Endeavour, 5, 35.

Marrian, G.F. and Beall, D.(1935). Biochem.J.29,1586.

x Marrian, G.F. and Haslewood,G.A.D. (1932). Biochem. J. 26, 1227.

Mason, H.L. and Hoehn, W.M.(1938,a). J.Amer. Chem. Soc. 60, 2566.

Mason, H.L. and Hoehn, W.M.(1938,b). J. Amer.Chem. Soc. 60, 2824.

Meyer, Hans (1938). Analyse und Konstitutionsermittlung organischer Verbindungen. 6th ed. Springer, Wien.

x Moffett, R.B. and Hoehn, W.M.(1944). J.Amer.chem. Soc. 66, 2098.

x Odell, A.D. and Marrian, G.F. (1938). J. biol. Chem. 125, 333.

x Olbrycht, T.M. (1942). Vet. Rec. 54, 81.

x Oppenauer, R. (1941). Hoppe-Seyl. Z. 270, 97. Chem. Abstr. (1943), 37, 673.

x Pincus, G. and Pearlman, W.H.(1943). Vitamins and Hormones, 1, 294.

x Prelog, V. and Führer, J.(1945). Helv. chim. Acta. 28, 583.

x Rea, C.E. (1940). Endocrinology, 26, 913.

Reich, H. and Reichstein, T. (1943). Helv. chim. Acta. 26, 562.

Reichstein, T.(1936). Helv. chim. Acta. 19, 1107.

--- (1937). Helv. chim. Acta. 20, 967.

Reichstein/

x Reichstein, T. and von Euw, J.(1938a,b). Helv.chim. Acta. 21, 1181, 1197.

Reichstein, T. and von Euw, J.(1941). Helv. chim. Acta. 24, 247E.

Reichstein, T. and Shoppee, C.W. (1943). Vitamins and Hormones. 1, 345.

Richter, J. and Gehring, K.(1935). Berl. tierärztl. Wschr. 51, 829.

Rosenheim, O. and Webster, T.A.(1943). Biochem.J. 37, 513.

Ruzicka, L., Huyser, H.W., Pfeiffer, M. and Seidel, C.F. (1929). Liebig's Ann. d. Chemie. 471, 25.

x Sala, S.L.(1935). Rev. sudamer. Endocr. 18, 325. (Brit.Chem.Abstr. 1936,A,753)

x Sandulesco, G. and Laboratoires francaises de Chimiotherapie. (1936). British pat. 485,388. (Brit. chem. Abstr. 1938, B, 1101).

x Sandulesco, G., Tchung, W.W. and Girard, A.(1933). C.R.Acad. Sci. Paris. 196, 137.

x Schachter, B.(1939). Ph.D. Thesis, University of Toronto.

x Schachter, B. and Marrian, G.F. (1938). J.biol. Chem. 126, 663.

x Schering, A.G. (1942). German pat. 719,368. (Chem. Abstr. (1943). 37, 2142).

x --- (1943). German pat. 732,813.(Chem. Abstr.(1944). 38, 838).

x Schering-Kahlbaum, A.G.(1932). Brit. pat. 391,686. (also German pat. 584,211). Chem.Zentr. 1933,II,1553.

x Schwenk, E. and Hildebrandt, F.(1932). Naturwissenschaften. 20, 658.

x Serringhaus, E.L. and St.John, R.(1943). J.clin. Endocrinol. 3, 98.

x Sholl, L.B. and Dersham, G.E.(1939). J.Amer.vet. med. Ass. 95, 507.

Shoppee/

Shoppee, C.W.(1940). Helv.chim. Acta. 23, 925.

Shoppee, C.W. and Reichstein, T.(1940). Helv.chim. Acta. 23, 729.

x Smith, E.R., Hughes,D., Marrian, G.F. and Haslewood, G.A.D. (1933). Nature, Lond. 132, 102?

Sorkin, M. and Reichstein, T.(1945). Helv.chim. Acta, 28, 875.

Sorkin, M. and Reichstein, T. (1946). Helv.chim.Acta, 29, 1218.

x Stammler, S. and Djatlowa, E. (1932). Klin.Wschr.11,422.

Steiger, M. and Reichstein, T. (1937). Helv.chim. Acta. 20, 821.

Steiger, M. and Reichstein,T. (1938,a). Helv.chim. Acta. 21, 546.

Steiger, M. and Reichstein, T.(1938,b). Helv.chim. Acta. 21, 828.

x van Stolk, D. and de Lenchere, R.L.(1937). C.R.Acad. Sci. Paris. 205, 395.

x van Stolk, D., Penau,H , de Lenchere, R.L. (1936). J.Pharm. Chim. Paris, 24, 249.

x Svensson. (1936). Skand. Vet. Ticskr. 26, 550.

Talbot, N.B., Ryan, J. and Wolfe, J.K. (1943). J. biol. Chem. 148, 593.

Tschesche, R. and Bohle, K.(1936). Ber.dtsch. chem. Ges. 69, 2497.

x Turner, V.H., Davis, C.D. and Hamblen, E.C.(1943). J.clin. Endocrinol. 3, 453.

x Weil, P.G. (1938). Proc. Soc. exp. Biol. N.Y. 38,503.

Westerfeld, W.W.(1942). J.biol. Chem. 143, 177.

Wintersteiner, O.(1937). Cold Spr. Harb.Sym. quant.Biol. 5, 25.

Wintersteiner/

x Wintersteiner, O., Schwenk, E., Hirschmann, H. and Whitman, B. (1936). J.Amer.chem. Soc. 58, 2652.

x Wintersteiner, O., Schwenk, E. and Whitman, B.(1935). Proc. Soc. exp. Biol. N.Y. 32, 1087.

Wolfe, J.K., Hershberg, E.B. and Fieser, L.F.(1940). J.biol. Chem. 136, 653.

x Zondek, B. (1930). Klin.Wschr. 9, 2285.

x --- (1931). Die Hormone des Ovariums und des Hypophysenvorderlappens. Berlin,Springer.

x --- (1934,a). Nature, Lond. 133, 209.

x --- (1934,b). Nature, Lond. 133, 494.

x --- (1934,c). Ark.Kem.Mrn.Geol. 11B, no.24.