On the Synthesis of Corticoids and Spectrophotometric Studies in the Steroid Hormone Group.

A Thesis

by

Hans L. Falk

Submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

McGill University

April 1947

TABLE OF CONTENTS

Part 1 A .

The part-synthesis of 12-keto-desoxycorticosterone acetate.

1. Introduction.

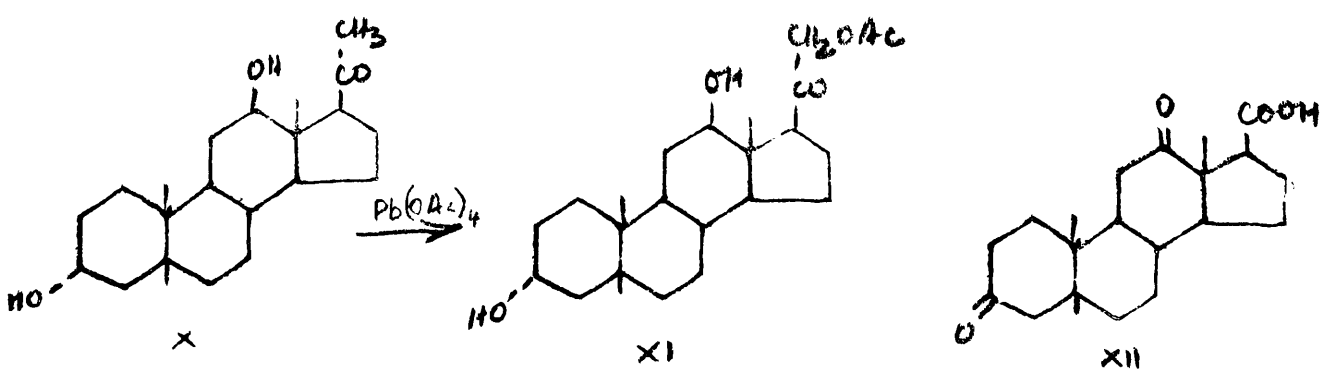
When the importance of the hormones of the adrenal cortex under conditions of stress had been established, the value of the hormones for the treatment of the injured and fatigued was recognized. The only hormone of the adrenal cortex, which is commercially available for therapeutic application, desoxycorticosterone acetate, however, has been found unsatisfactory.

Most adrenal cortical hormones possess an oxygen atom in the 11-position, which endows them with gluconeogenetic activity. Owing to the tedious and expensive methods for the isolation of the hormones from adrenal cortical tissues, the synthesis of the compounds from the bile acids has been investigated. Attention was focussed on the transposition of the 12-oxygen atom to the 11-position, and several successful methods were developed. (389-391, 409-412) The large number of reactions involved and the poor yields make commercial production economically unfeasible.

An investigation of the gluconeogenetic activity of the 12-oxygen analogues of adrenal cortical hormones became of great interest, because the synthesis of these compounds from desoxycholic acid eliminates the costly and laborious transposition reactions.

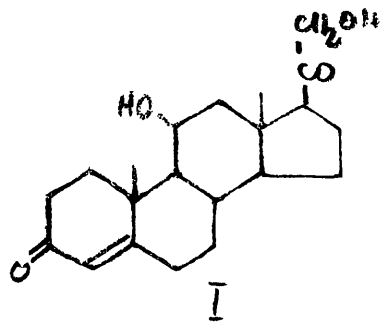
The part-synthesis of 12-keto-desoxycorticosterone acetate had been undertaken in this laboratory by D. Sainte-Marie (392), who investigated the action of lead tetraacetate on pregnane-3,12-diol-20-one and obtained pregnane-3,12-diol-21-acetoxy-20-one amongst other reaction products. Fuchs and Reichstein (393) built up the ketol sidechain from etiodesoxycholic acid and prepared the 12-keto, 12-hydroxy and 12-acetoxy analogues of cortical hormones. All three compounds were found inactive in the Everse-de Fremery test (399). The 12-acetoxy homologue was also tested in the Anti-Insulin reaction of Jensen and Grattan (398) and four mg. doses per mouse were found to be inactive.

The gluconeogenetic activity of these compounds had not been adequately investigated, and the part-synthesis of a batch, large enough for this purpose, was undertaken.

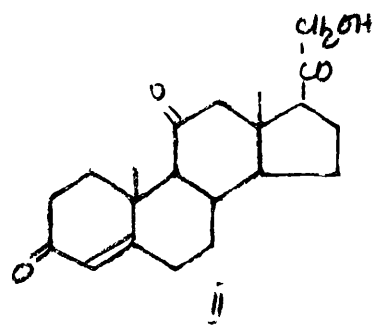


X XI XII

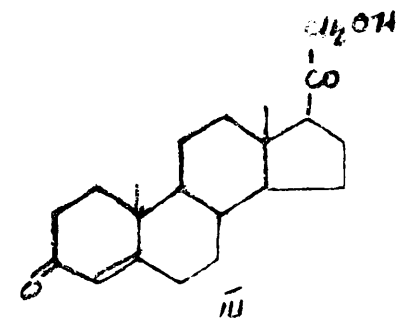
Adrenal Cortex Hormones.



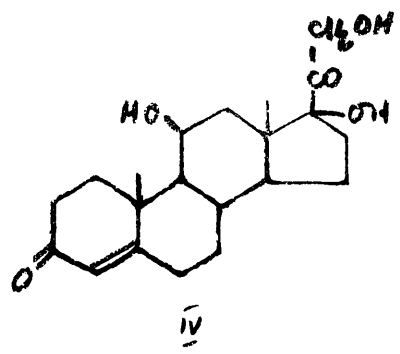
I



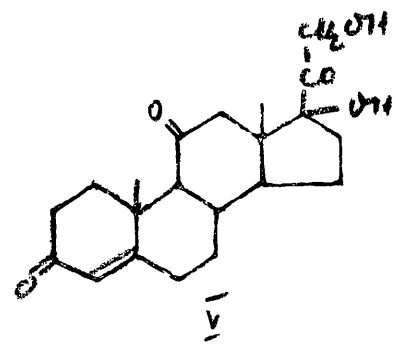
II



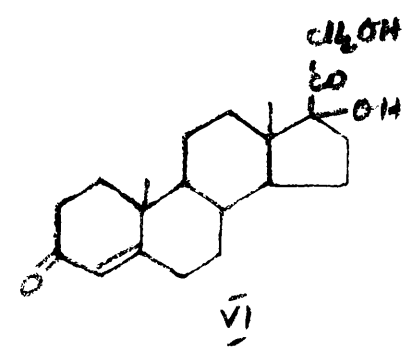
III



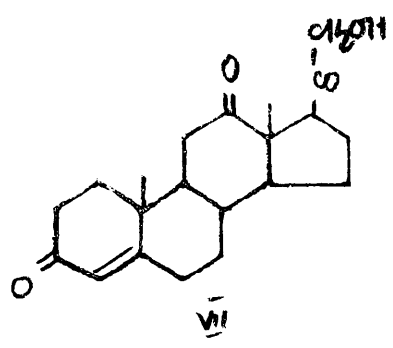
IV



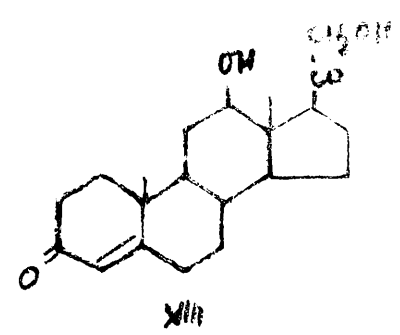
V



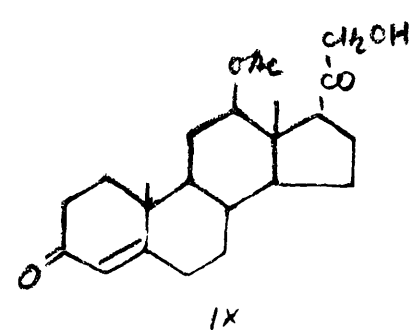
VI



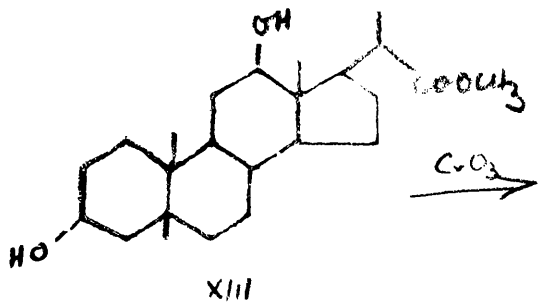
VII



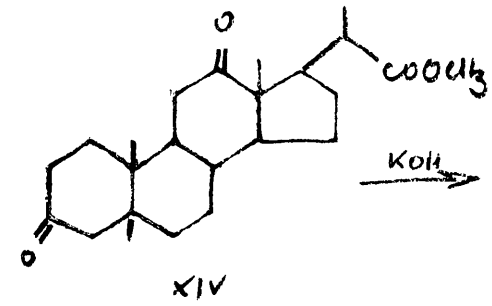
VIII



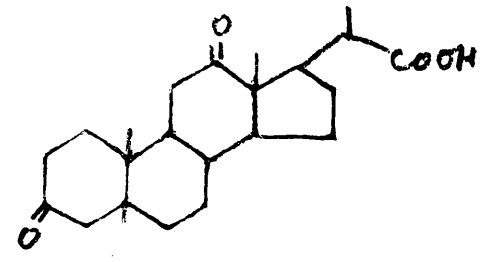
IX



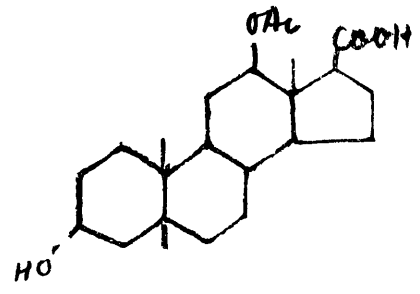
XIII



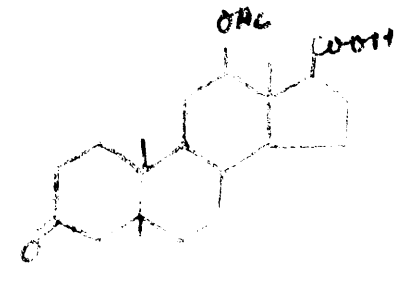
XIV



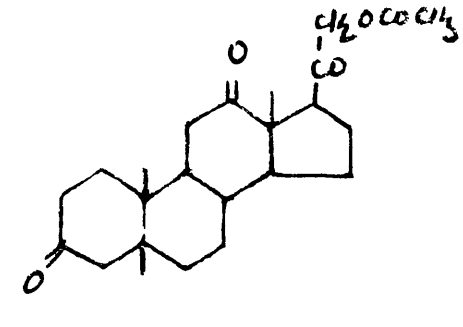
XV



XVI



XVII



XVIII

2. Discussion.

In an attempt to prepare pregnane-21-acetoxy-3,12,20-trione (XVIII) from 3,12-diketo-etiocholanic acid (XV) D. Sainte-Marie (392) obtained dark coloured oils, from which only small amounts of the desired crystals could be isolated, while Fuchs and Reichstein (393) prepared the corresponding ketol (XI) in good yield from 3,12-diacetoxy-etiocholanic acid (XX).

To investigate this unexpected difference in the behaviour of the 3,12-diketo and the 3,12-diacetoxy acid, model experiments were conducted with the cheaper bisnor-desoxycholic acid. Methyl bisnordesoxycholate was oxidized with chromic acid to the corresponding diketo compound (XIV), saponified, and the resulting acid was purified by recrystallisation and thoroughly dried in a vacuum-desiccator.

In the conversion of the acid (XV) to the acid chloride with thionyl chloride, the solution soon turned from yellow to red and purple. As this coloration had not been described by Fuchs, it appeared, that the keto compound was unsuitable for this reaction. The product, however, was treated with diazomethane, and the resulting oil was heated with acetic acid. Only brown, amorphous solids, showing slight reducing properties, could be isolated.

The experiment was repeated with the same results on another batch of diketo-bisnorcholanic acid, taking great care to have all reagents and solvents perfectly pure and dry. A comparison was therefore made of the action of thionyl chloride on 3,12-diketo and 3,12-diacetoxy-bisnorcholanic acid under identical conditions. Whereas the solution of the diketo compound rapidly turned red, only a slight yellow coloration of the 3,12-diacetoxy-bisnorcholanyl chloride was observed. The compound was treated with diazomethane, saponified and decomposed with acetic acid. The product could not be crystallized, even after chromatographic separation, and on chromic acid oxidation only colourless oils were obtained.

As the presence of a ketone group appears to interfere with the acid chloride formation, it was investigated, whether the 12-keto group alone was responsible. The 3-hydroxy-12-acetoxy-etiocholanic acid (XVI) was oxidized to the 3-keto compound (XVII), which on treatment with thionyl chloride was observed to turn purple. The conclusion was reached, that a 12-keto group was not essential for this untoward reaction.

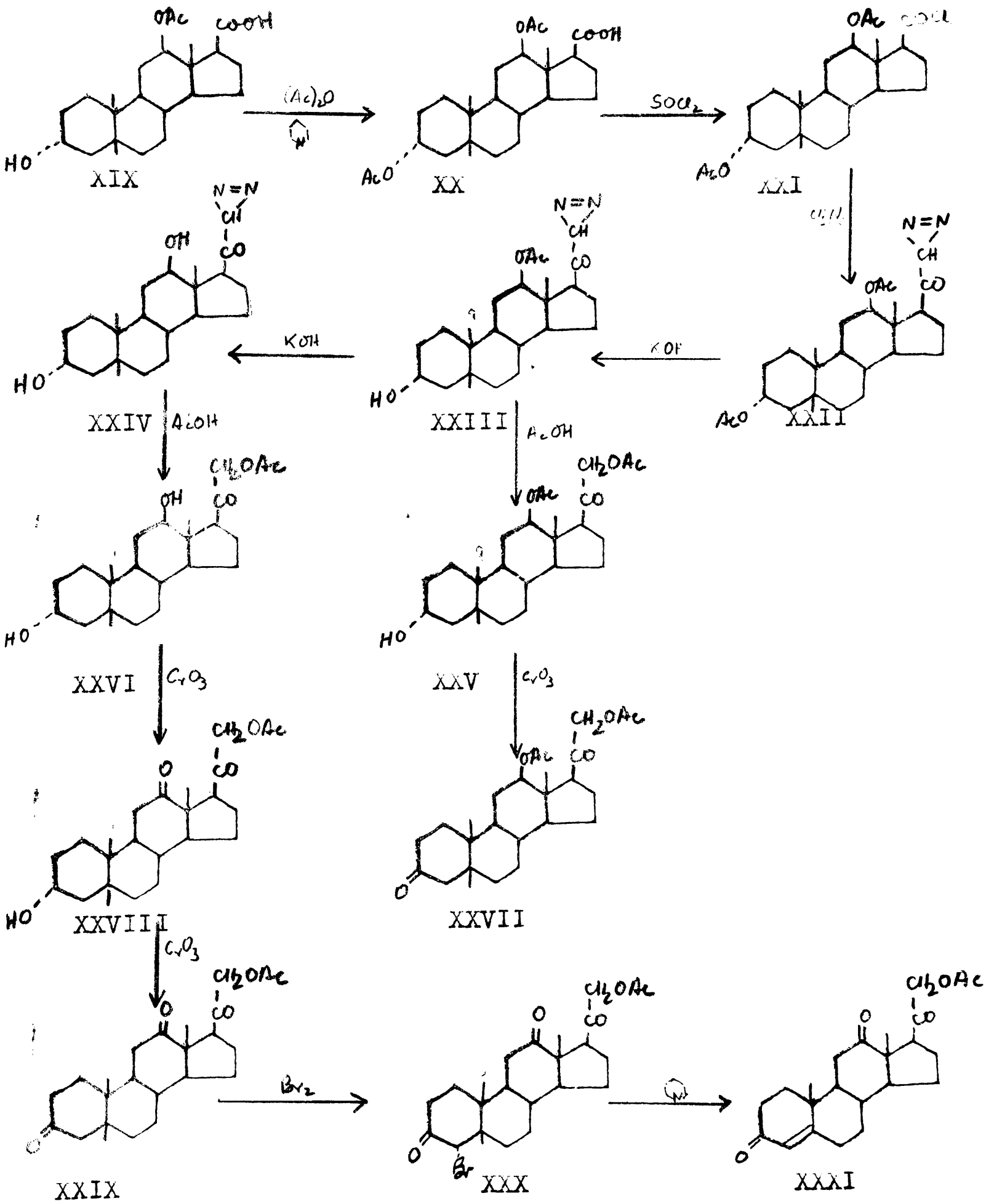
After these preliminary experiments, the part-synthesis of 12-keto-desoxycorticosterone acetate (VII) from 3-hydroxy-12-acetoxy-etiocholanic acid (XVI) was

undertaken. A trial run was made on one g. of acid (XIX), following the directions of Fuchs and Reichstein. On acetylation the anhydride was formed which, on hydrolysis with aqueous acetic acid, gave rise to the diacetate (XX).

The pure, dry crystals (XX) were added to a solution of thionyl chloride forming a light yellow solution. The acid chloride (XXI) was not purified, but was treated with diazomethane, saponified, and decomposed with glacial acetic acid. On chromatography crystals were obtained, which showed the characteristic melting point of pregnane-21-acetoxy-3,12-diol-20-one (XXVI). The preparation of the compound was repeated a number of times without modification, except for the immediate hydrolysis of the etio acid anhydride without separation. The yields varied from 67% on a small scale reaction to 60% on larger ones.

Oxidation with chromic acid gave rise to the diketo compound (XXIX) in quantitative yield, when only 100 mg. amounts were treated. In the case of larger batches, the yield dropped to 77% and crystals of 3-ol-21-acetoxy-pregnane-12,20-dione were also isolated.(XXVIII)

Bromination (404,405) did not present any difficulties except in one case, where ultraviolet radiation and strong sunlight had to be used to start the reaction.


OAc
COOH
HO
XIX
(Ac)2O
Py
AcO
XX
SOCl2
XXI
CH2N2
N=N
CH
CO
XXII
KOH
XXIII
XXIV
AcOH
CH2OAc
OH
XXVI
CrO3
XXVIII
XXV
XXVII
Br2
XXIX
XXX
XXXI

The yield amounted to 60%. In subsequent brominations, the product (XXX) was not purified but debrominated directly with pyridine. The desired end product (XXXI) was obtained in 58% yield. On debromination of the purified bromo compound 69% yields of crystalline material (XXXI) were reached. Crystalline by-products were separated by chromatography. Identification of the compounds, however, was not attempted, owing to the large number of possible side reactions.

Pure crystals (XXXI) were tested by Dr. Venning for gluconeogenetic activity. When examined by the glycogen deposition test of Reinecke and Kendall (400), 12-keto-desoxycorticosterone acetate was found to be toxic at a dose level of 0.5 to 5 mg. and completely inactive in a 0.2 mg. dose. The compound was also found to be inactive by the Ingle test (401). From these and Fuchs and Reichstein's results it was concluded, that the compound was inactive, and toxic in higher dosis.

3. Experimental work.

1. Oxidation of methyl bisnordesoxycholate.

The ester (XIII) (10.0327 g.) was dissolved in glacial acetic acid(100 ml.), and chromic acid (7.1 g. in 80 ml. 80% acetic acid) was added dropwise to the cooled solution. After standing for two hours at 0°C, the solution

was diluted to ten volumes with water and extracted with ether (3x600 ml.). The ether extracts were washed with 10% sodium carbonate solution (2x200 ml.),washed neutral with water, dried with sodium sulphate and taken to dryness. The crystals melted at 140-142°C.

All melting points were observed under a cover slip on a microscope slide heated on the stage of the Kofler - Hilbck micro melting point apparatus. The values recorded are corrected,the limits defining the temperature of first appearance of liquid and of complete clearing of the melt.

2. Saponification of methyl 3,12-diketo-bisnorcholanate.

The diketo ester (XIV)(7.825 g.) was dissolved in 36 ml. methanol and 18 ml. 6N potassium hydroxide solution, and refluxed on the steam bath for three hours. The solution was then cooled, diluted with ten volumes of water, washed once with ether (100 ml.),acidified and filtered. The white residue was crystallized from acetone. m.p.263-264°C. A quantitative yield was obtained.

3. The action of thionyl chloride on the diketo acid.

After thorough drying in a desiccator, the acid (XV)(9 g.) was added to 46 ml. thionyl chloride (distilled over quinoline and boiled linseed oil), which was cooled in an ice salt freezing mixture. The solution was kept under

anhydrous conditions at 0°C for 30 minutes, and 20 hours at room temperature. A colour change from yellow to orange, red and purple was observed. The solvent was removed under vacuum, using as little heat as possible. The dark brown residue was not purified.

4. The action of diazomethane on the product.

Nitrosomethylurea was prepared according to directions given in Organic syntheses (408), from which diazomethane was produced by the method described in the same volume (408). Great care was taken to prevent the escape of highly toxic diazomethane.

The oily residue was dissolved in 50 ml. dry benzene and added to 80 ml. dry ether containing diazomethane (prepared from 30 g. nitrosomethylurea) at a temperature of -15°C. (salt ice freezing mixture). The solution was kept two hours at 0°C, and 16 hours at room temperature under anhydrous conditions in a fume cupboard. The ether and excess diazomethane were distilled at room temperature under vacuum, with an acetic acid trap for escaping diazomethane. When the benzene had been removed under vacuum at 40°C, a brown oil remained which was not purified.

5. The decomposition of the product with acetic acid.

The residue was dissolved in 100 ml. glacial acetic acid and heated on the steam bath for 55 minutes. On cooling

a black solid settled out. After the solvent had been removed under vacuum on the steam bath, crystallisation of the residue was attempted unsuccessfully. A number of fractions showed reducing properties with the silver diamine reagent.

The reactions were repeated on another batch of diketo-bisnorcholanic acid, and great care was taken to use perfectly pure and dry reagents and solvents (407). Identical results were obtained. The dark brown residue did not yield any crystalline material after chromatography.

6. Comparison of the action of thionyl chloride on the diketo and diacetoxy-bisnorcholanic acid.

3,12-diketo-bisnorcholanic acid (2.238 g.) and 3,12-diacetoxy-bisnorcholanic acid (2.31 g.) were dropped separately into 11.5 ml. thionyl chloride solutions and kept at 0°C under anhydrous conditions. The solution containing the diketo acid turned dark red, while the other solution remained light yellow. The thionyl chloride was removed from the solution, containing the diacetoxy compound, under vacuum at 40°C. The oily residue was treated with diazomethane and extracted as described above. The oil was dissolved in methanolic potassium hydroxide (1.5 g. in 38 ml. 90% methanol) and saponified at room temperature for 22 hours. To the red solution potassium bicarbonate (2.75 g. in 55 ml. water) was added and the methanol was removed under

vacuum at 20°C. The mixture was then extracted with ether, (3x100 ml.) and the ether was washed (3x50 ml.), dried with sodium sulphate and distilled. The yellow oil was dried thoroughly and decomposed with glacial acetic acid, as previously described. On chromatography of the residual oil, light yellow oily fractions were eluted. They were oxidized with chromic acid yielding colourless uncrystallizable oils.

7. Oxidation of 3-hydroxy-12-acetoxy-etiocholanic acid.

The monoacetate (223 mg.) was dissolved in glacial acetic acid (5 ml.), and chromic acid (0.3 ml. 4 N) was added dropwise to the cooled solution with constant shaking. After 3 hours at room temperature, an additional 0.15 ml. 4N chromic acid solution was added and allowed to react for 30 minutes. The solution was diluted with 10 volumes of water, and the crystalline precipitate was filtered. (207 mg.), m.p. 239-241°C.

8. The action of thionyl chloride on 3-keto-12-acetoxy-etiocholanic acid.

The 3-keto acid (XVII)(22.7 mg.) was added to the thionyl chloride (0.25 ml.) at 0°C. On standing in ice under anhydrous conditions the solution turned orange, red and purple after two hours.

9. Acetylation of etiodesoxycholic acid-12-acetate.(XIX).

The monoacetate (XIX)(5 g.) was thoroughly dried in a vacuum desiccator, dissolved in 50 ml. acetic anhydride and allowed to stand for 16 hours at room temperature under anhydrous conditions. The solvent was then removed under vacuum, and the residue was taken up in ether (200 ml.) and extracted with ice cold 5% sodium carbonate solution (3x60 ml.). The carbonate extracts were quickly acidified with dilute hydrochloric acid, to prevent hydrolysis of the 3-acetate group, extracted with ether (3x60 ml.), and the combined ether solutions were washed neutral (3x30 ml.) and taken to dryness. The oily residue weighed ten mg. The neutral fraction (6.01 g.) was reclaimed from the washed ether solution, treated with 23 ml. acetic acid and 10.5 ml. of water and heated on the steam bath for two hours. The solvents were removed under vacuum, the residue dissolved in ether (200 ml.) and was separated into an acidic and neutral fraction with 5% sodium carbonate solution, as described above. In the acid fraction crystals were obtained on acidification of the carbonate extracts, which were recrystallized from ether-petrol ether.m.p.196-198°C. The neutral fraction (0.217 g.of oil) did not yield acidic material on repeated hydrolysis with aqueous acetic acid.

In subsequent acetylations the anhydride was not separated, but was immediately hydrolyzed. The amount of

of neutral, unhydrolyzable oil varied from 0.5% - 4%. Pure crystalline diacetate was obtained in 75% yield. The mother liquors, reacetylated, yielded more crystalline diacetate.

10. The action of thionyl chloride on 3,12-diacetoxy-etio-desoxycholic acid (XX - XXI).

Thoroughly dried crystals of the diacetate (XX) (5.8634 g.) were added to 30 ml. purified thionyl chloride (407) at -15°C (salt ice freezing mixture), and the solution was allowed to stand 30 minutes at 0°C and 20 hours at room temperature under anhydrous conditions. The solvent was then removed under vacuum below 50°C. A light yellow oil was obtained, which was not purified.

11. The action of diazomethane on 3,12-diacetoxy-etiocholanyl chloride. (XXI - XXII).

The oil (XXI) was dissolved in 50 ml. pure benzene (407) and added to the ether solution (407) of diazomethane (prepared from 18.54 g. nitrosomethylurea) at - 15°C. A flocculent precipitate settled out. The mixture was then left standing for 2 hours at 0°C and 20 hours at room temperature under anhydrous conditions in the fume cupboard. Excess diazomethane and ether were distilled with the necessary precautions below 40°C, and the benzene was taken off at 40°C under suction. The yellow, oily residue was not purified.

12. Saponification of pregnane-21-diazo-3,12-diacetoxy-20-one. (XXII - XXIV).

The oil (XXII) was saponified with methanolic potassium hydroxide (3.91 g. in 92 ml. 90% methanol) on standing for 20 hours at room temperature. Potassium bicarbonate (7 g. in 140 ml. of water) was added to the red solution, and most of the methanol was removed under vacuum at 20°C. The precipitate was extracted with ether (3x60 ml.), and the ether was washed neutral with water and distilled. An oil (5.6619 g.) was obtained and thoroughly dried on the high vacuum at 45°C.

13. The action of glacial acetic acid on pregnane-21-diazo-3,12-diol-20-one. (XXIV - XXVI).

Glacial acetic acid (32.5 ml., purified by distillation from lead tetraacetate and chromic acid) was added to the oily residue (XXIV) and the solution was heated on the steambath for 40 minutes. The liberated nitrogen gas was collected over water in an upturned measuring cylinder and measured, allowance being made for the displacement of water by the expansion of gas inside the heated flask. When the evolution of nitrogen had ceased — 315 ml. nitrogen gas had been given off (95% of theory) —, the acetic acid was removed on the water bath under vacuum, leaving an oily residue (5.8764 g.) which crystallized partly.

14. Chromatography of pregnane-21-acetoxy-3,12-diol-20-one.

A column of alumina (Harshaw, acid washed and re-activated) was prepared (30x the weight of adsorbate in a column, its height 5x its width) and washed with petrol ether. The oil (XXVI) was dissolved in a minimum amount of dry benzene, and dry petrol ether was added to incipient turbidity. Fractions were eluted with various solvent mixtures, as outlined below. 50 ml. solvent mixtures were used for each fraction.

Fraction No.	Eluant	Eluate mg.	nature	m.p.
1 - 4	benzene 100%	33.5	oil	
4 - 7	bz.: ether 40:1	8.6	"	
8 -11	bz.: ether 20:1	23.8	"	
12 -13	bz.: ether 10:1	22.2	"	
14 -15	bz.: ether 4:1	33.4	"	
16 -17	bz.: ether 2:1	63.0	"	
18 -21	bz.: ether 1:1	854.8	"	
23 -28	bz.: ether 1:1	1012.5	crystals	101°,149-151°C.
29 -48	ether 100%	3086.1	"	" " "
49 -50	ether : $CHCl_3$ 1:1	42.3	oil	
51 -52	chloroform 100%	16.7	"	
53 -58	ethyl acetate: $CHCl_3$: CH_3OH 1:2:1	1137.3	"	
		total 5606.9	"	

The crystals, eluted in fractions 23 to 48, were dried over phosphorus pentoxide on the high vacuum at 80°C. They showed the same double melting point and were identified as compound XXVI (393). On a small batch (1 g.) 67.2% yield was obtained. On larger ones (6 - 7 g.) yields of 58 to 62% could only be reached. In addition pregnane-3-ol-12,21-di-acetoxy-20-one (XXV) was obtained as an oil in 6% yield,

which was identified after chromic acid oxidation to crystalline pregnane-12,21-diacetoxy-3,20-dione,m.p.118-123° (XXVII) Compound XXV was eluted with solvent mixtures benzene : ether 20 : 1.

15. Oxidation of pregnane-21-acetoxy-3,12-diol-20-one (XXVI).

Chromic acid (133.4 g.) was diluted to one litre with distilled water,and its strength was checked by titration with 0.1 N sodium thiosulphate solution (standardized with an exact 0.1 N potassium biiodate solution ($KIO_3.HIO_3$)) in the presence of potassium iodide and acetic acid. A dilute starch solution served as indicator. The chromic acid solution was 4 N.

A solution of 98.6 mg crystals (XXVI) in 3 ml. glacial acetic acid was prepared, and 0.26 ml. 4 N chromic acid solution was added dropwise with shaking to the ice cold solution. After standing for three hours at room temperature, the test for the presence of chromic acid did not give a violet colour. (two ml. dilute sulphuric acid, one ml. ether and a few drops hydrogen peroxide solution in a small test tube show on addition of chromic acid a violet streak.) An additional 0.13 ml. 4 N chromic acid solution was added and allowed to react for one hour at room temperature. Excess chromic acid was detected. The solution was diluted with ten volumes water, and the precipitate was extracted

with ether (3x20 ml.). The ether was washed with dilute hydrochloric acid (2x10 ml.),with ice cold 5% sodium carbonate solution (2x10 ml.) and with water until neutral,dried with sodium sulphate and distilled. Colourless,thermolabile crystals were obtained,m.p.189-191°C, showing no depression of the mixture melting point with pregnane-21-acetoxy-3,12,20-trione.

On oxidation of 100 to 250 mg. batches quantitative yields were obtained, while with larger quantities yields dropped to 73 - 77%. Oxidation was not complete and on chromatography of the mother liquors crystals of compound (XXIX) as well as crystals of pregnane-21-acetoxy-3-ol-12,20-dione (XXVIII) were eluted. The crystals (XXVIII) on chromic acid oxidation gave rise to pregnane-21-acetoxy-3,12,20-trione.

Fraction No.	Eluant 50 ml.	Eluate mg.	nature	m.p.	formula
1 - 2	Pet.E.:Bz.1:1	46.4	oil		
3 - 14	benzene 100%	714.3	crystals	189-191°	XXIX
15 - 19	bz.:ether 50:1	86.5	"	148-151°	XXVIII
20 - 21	" 20:1	47.0	"	"	"
22 - 23	" 8:1	40.0	"	"	"
24 - 25	" 4:1	52.1	"	"	"
26 - 27	" 2:1	238.4	oil		
28 - 35	" 1:1	943.6	"		
36 - 44	Ether 100%	374.5	"		
45 - 48	Ether:$CHCl_3$ 1:1	36.7	"		
49 - 50	$CHCl_3$ 100%	8.8	"		
51 - 52	ethyl acetate: $CHCl_3$:CH_3OH 1:2:1	189.6	"		
	total	2722.9			

16. Bromination of pregnane-21-acetoxy-3,12,20-trione. (XXIX)

Approximately 12 g. bromine were dissolved in 100 ml. glacial acetic acid. One ml. of this solution was neutralized by 15.25 ml 0.1015 N sodium thiosulphate solution (restandardized with potassium biiodate solution) The bromine solution, therefore, contained 12.38 g. bromine per 100 ml. solution.

Compound XXIX (786.8 mg.) was dissolved in glacial acetic acid (8 ml.) and 0.1 ml. bromine solution was added. After 30 seconds the solution turned colourless, and 2.55 ml. bromine solution (1.05 equivalents) was added from a burette dropwise with cooling and shaking. When the solution was decolorized, the acetic acid was removed at 30°C under high vacuum and the residual oil was crystallized from ether. m.p. 186-190°C. On recrystallisation from methanol, the crystals showed a double melting point at 144° and 176°C and decomposed on standing in the open. Recrystallized from ether, their melting point was again 190-192°C.

The mother liquors were dissolved in acetic acid (10 ml.) and reduced with zinc at 20°C for 4 hours. The solution was diluted to 10 volumes with water, extracted with ether (3x30 ml.), and the ether was washed with iced 5% sodium carbonate solution (1x20 ml.), washed neutral, dried and distilled. Crystals of pregnane-21-acetoxy-3,12,20-trione, (XXIX) were isolated, m.p. 190-191°C.

In one bromination experiment a 60% yield was obtained. On a larger batch the yield of the bromo compound (XXX) was considerably smaller, and the isolation of the unstable bromo compound was eliminated in later trials. The bromination product was directly debrominated after thorough desiccation.

17. Debromination of pregnane-21-acetoxy-4-bromo-3,12,20-trione. (XXX).

The bromo compound (XXX)(948.4 mg.) was dissolved in anhydrous pyridine (10 ml.) and gently refluxed for five hours at 115 - 120°C on an oil bath, under a stream of dry nitrogen. The pyridine was removed at 70°C under high vacuum and the oily residue crystallized on standing. The crystals were dissolved in chloroform (10 ml.). The solution was diluted with 4 volumes of ether, washed with 10% hydrochloric acid solution (3x10 ml), with ice cold 5% sodium carbonate solution (3x10 ml.) and with water until neutral, dried with sodium sulphate and taken to dryness at 45°C. Crystals were obtained, which melted from 180-190°C. They were chromatographed as shown on page 21 and recrystallized from methanol. The molar extinction coefficient at λ_{max} 240 mμ of the crystals, m.p. 184-6° C, was calculated to be 16600 indicating 100% pure crystals of 12-keto-desoxycorticosterone acetate. (XXXI).

The yield of the reaction amounted to 69% (XXXI). When the bromo compound was not isolated, the yield was only 58%, but 17.4% crystalline unidentified material was also

obtained. As the number of possible bromination and debromination products is large, identification of the chromatographed side-reaction-products was not attempted.

Chromatogram of debromination product.

Fraction No.	Eluant 20 ml.	Eluate mg.	nature	m.p.	formula
1 - 5	Pet.E.:Bz 1:1	17.5	crystals	174-89°	?
6 - 8	" 1:3	185.2	"	179-97°	?
9 -10	" "	20.4	"	191-210°	?
11 -17	" "	185.4	"	181-184°	XXXI
18 -20	Benzene 100%	65.3	"	"	"
21 -24	Bz.:Ether 20:1	88.7	"	"	"
25 -28	" 10:1	86.3	"	"	"
29 -32	" "	11.0	oil		
33 -36	" 4:1	10.3	"		
37 -38	" 1:1	7.7	"		
39 -40	Ether 100%	5.0	"		
41 -42	$CHCl_3$ "	6.7	"		
43 -44	ethyl acetate: $CHCl_3$:CH_3OH 1:2:1	29.6	"		
	total	698.7	"		

Chromatogram of crystalline side products.

Fraction No.	Eluant 10 ml.	Eluate mg.	nature	m.p.
1 - 6	Pet.E.:Bz 1:1	4.7	oil	
7 - 8	" 2:3	4.9	needles	56-62°
9 - 11	" 1:3	8.4	crystals	191-204°
12 - 15	" 1:9	31.2	"	184-195°
16 - 18	benzene 100%	47.4	"	214-219°
19 - 22	bz.:ether 9:1	1112	"	203-218°
23 - 26	" 4:1	59.2	"	174-192°
27 - 28	" 2:1	4.2	oil	
29 - 30	" 1:1	8.9	"	
31 - 32	ether 100%	13.8	"	
33 - 34	$CHCl_3$ "	4.0	"	
35 - 36	ethyl acetate: $CHCl_3$:CH_3OH 1:2:1	3.1	"	
	total	206	"	

18. Bioassay of 12-keto-desoxycorticosterone acetate. (XXXI).

The compound XXXI was tested by Dr. Venning for gluconeogenetic activity by the glycogen deposition test of Reinecke and Kendall (400). The compound was injected in 7 equal doses at hourly intervals into adrenalectomized mice, whose liver glycogen had been depleted. The livers of these animals were then examined for glycogen. The results are combined in a table.

Compound	dose mg.	number of animals	observations or glycogen deposition/ 100 g. mouse
XXXI	5	6	all died after 4 injections
XXXI	0.5	6	2 " " 2 " 1 66 mg 1 66 mg sick 1 1 mg sick 1 14 mg
"E"	0.022		66 mg
"A"	0.070		66 mg
----	----		<10 mg

Compound XXXI was rechromatographed, to remove traces of toxic bromo compounds, and again tested by Dr. Venning. No impurities could be discovered.

0.5 mg XXXI proved toxic for 10 out of 10 animals.

0.2 mg XXXI did not cause glycogen deposition in any of the test animals.

The compound was also tested by the Ingle test. Three to 7.5 mg. doses were found to be inactive.

Part 1 B.

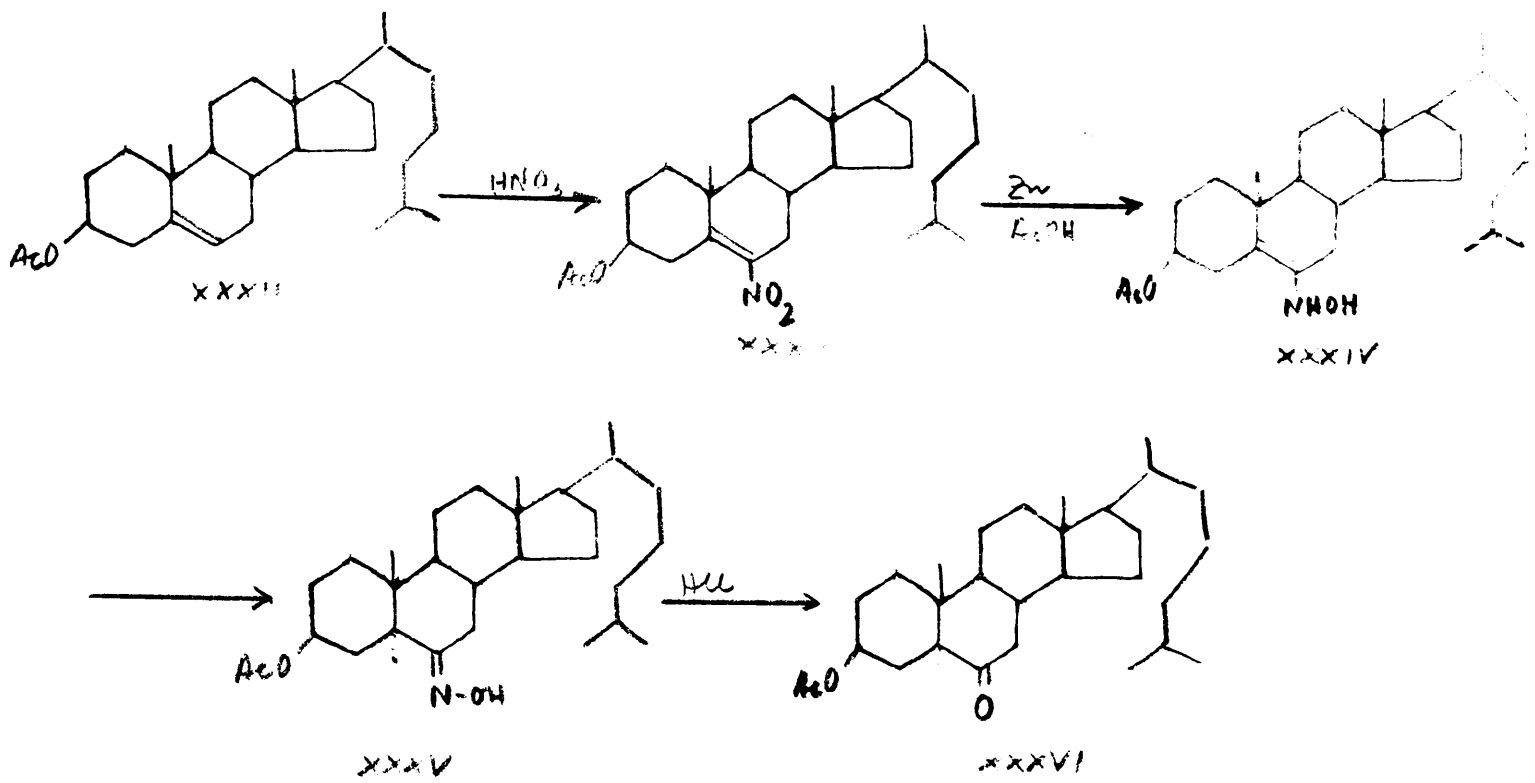
The Nitration of Δ 9:11-lithocholenic acid.

1. Introduction.

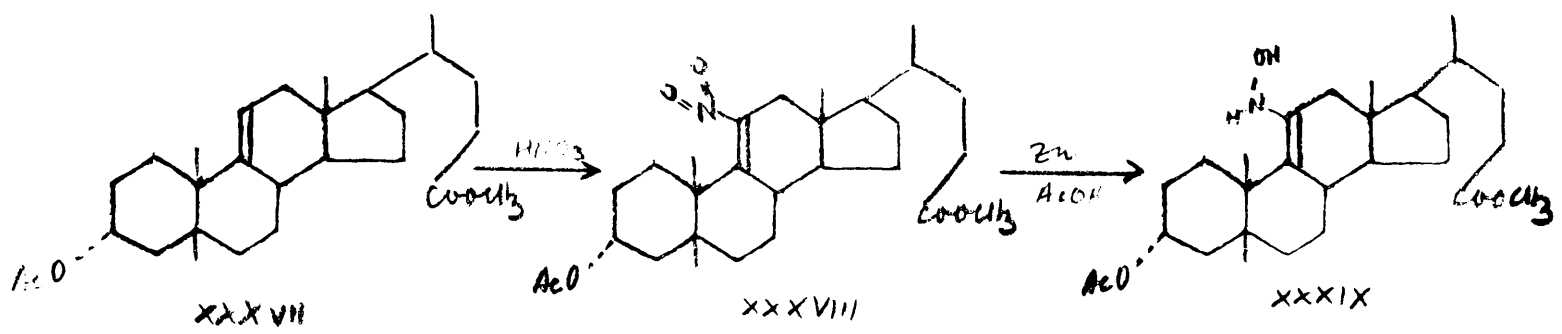
Since the 12-keto analogue of dehydrocorticosterone acetate proved to be inactive and toxic, attention was turned to the problem of transposing the oxygen atom from the 12 to the 11-position, where its presence is essential for gluconeogenetic activity of adrenal cortical hormones.

The transposition was accomplished by Reichstein (389), Kendall(391), Gallagher(409), Wallis(410), Sarret(411), and Wintersteiner(412), and corticosterone and Kendall's compound "E" has actually been prepared from desoxycholic acid. The number of steps involved and the limited yields of certain intermediate compounds, however, have prevented the commercial preparation of these hormones.

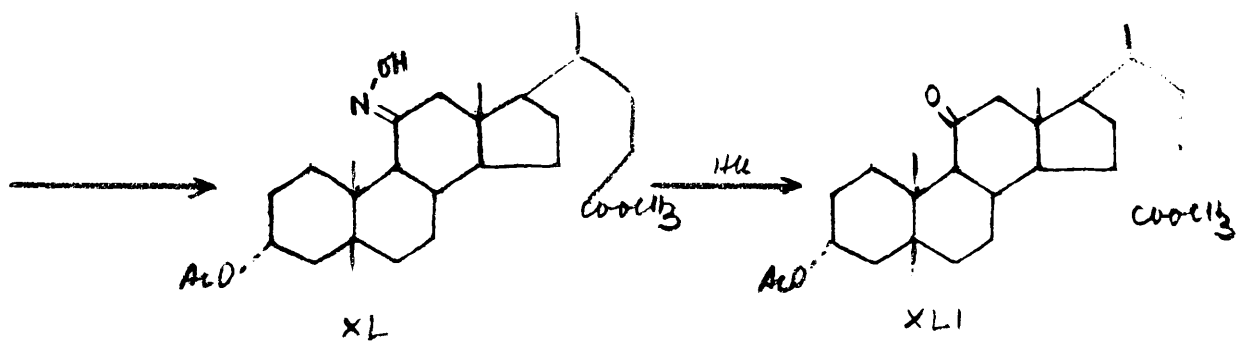
A nitration reaction, aiming at the introduction of an oxygen atom in the 11-position, was mentioned by Wallis (441), but the results of his investigation were not published. As the nitration of cholesterol acetate to 6-nitro-cholesterol acetate leads on hydrolysis after reduction to 6-keto-cholesterol acetate (Heilbron (440)), interest in the nitration of the 9:11 double bond in steroids persisted.



XXXIII XXXIV XXXV XXXVI



XXXVII XXXVIII XXXIX



XL XLI

The action of nitric acid and the oxides of nitrogen on olefinic double bonds has been studied by a number of investigators (443-447). A thorough analysis of the nitration products of olefinic compounds was made by Michael and Carlson (438,439) on methyl propene, trimethyl ethylene and tetramethyl ethylene. Aside from a number of addition products, they obtained a substitution product in 5 to 12% yield. Montignic (442) described the preparation of dinitro-cholesterol with concentrated nitric acid and Heilbron, as mentioned above, obtained the 6-nitro-cholesterol acetate in good yield.

The second step, the reduction of the nitro group to the oxime, has been described for a variety of nitro compounds by the use of stannous chloride and hydrochloric acid or zinc in acetic acid. (Houben (452)).

Hydrolysis of the oxime to the corresponding ketone (by refluxing with alcoholic hydrochloric acid) was described by Houben (452) and others (448-451). As all three reactions, involved in this investigation, were found to be widely applicable to organic compounds, including cholesterol and its acetate, the action of nitric acid on Δ9:11-lithocholenic was studied.

2. Discussion.

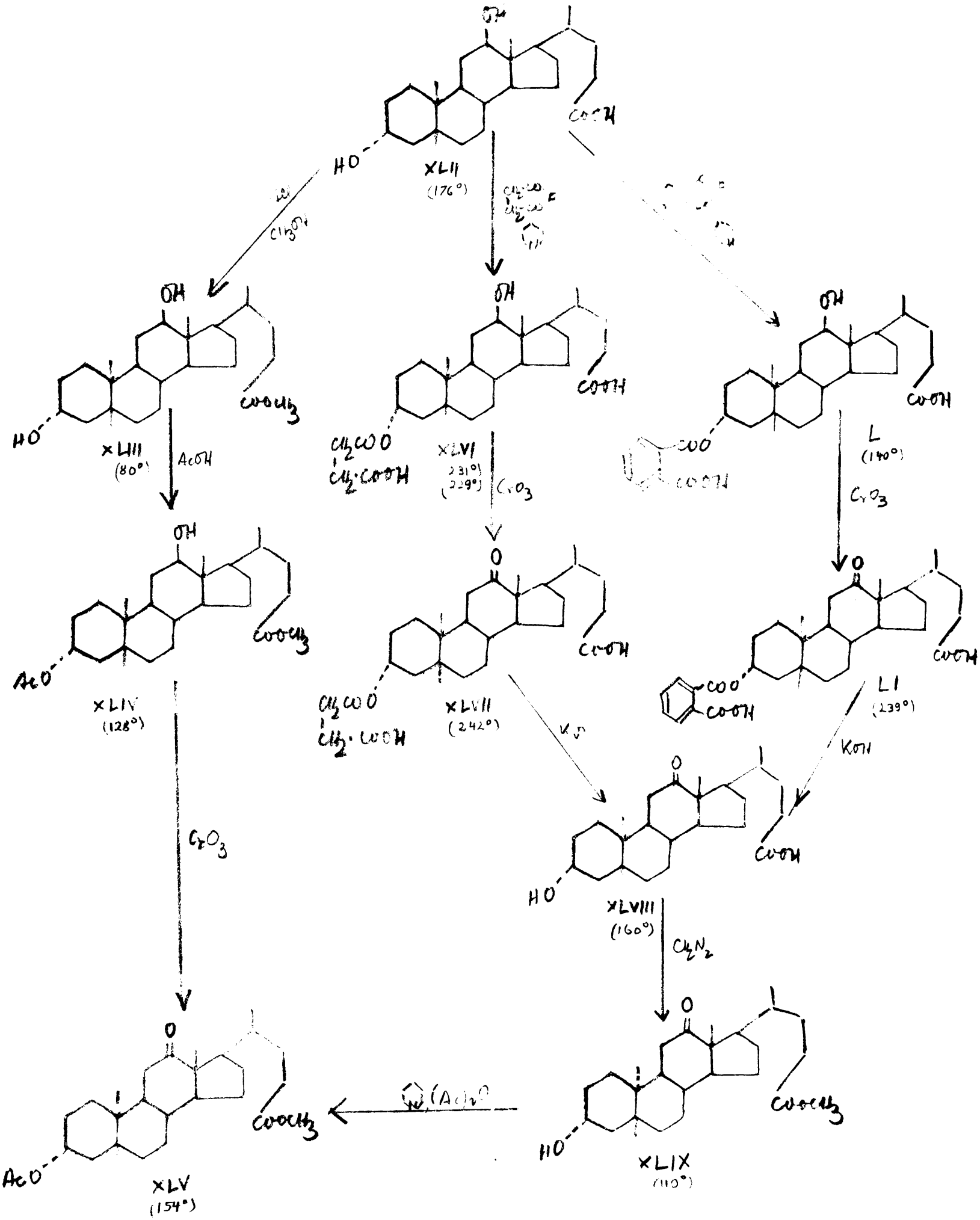
To investigate the action of nitric acid on a 9:11 double bond, Δ9:11-lithocholenic acid was prepared from desoxycholic acid. A number of methods for the formation of 3-hydroxy-12-keto-cholanic acid (XLVIII) were found in the literature. The partial oxidation of desoxycholic acid (XLII) with chromic acid, as described by Kashima(414), was not attempted. The oxidation of 3-acetoxy-12-hydroxy-cholanic acid(obtained by acetic and hydrochloric acid treatment of desoxycholic acid) appeared to be more reliable.(Wieland(417), and others(418,419)). The reaction product was not extracted, but directly oxidized with chromic acid as described by Reichstein(418). Methyl 3-acetoxy-12-keto-cholanate was obtained by this reaction, but showed a melting point 10 degrees lower than that given in the literature. On reacetylation the melting point remained unchanged. When the crystals were recrystallized from ether instead of methanol, the characteristic melting point, 152-154°C was observed.

Meanwhile a different method, yielding pure 3-hydroxy-12-keto-cholanic acid according to the literature(415,416), had been investigated. The C_3-semi-succinate can be isolated after treatment of desoxycholic acid with succinic anhydride and pyridine. Without application of heat, however, the reaction did not go to completion, while with heating on the steam bath a dark coloured, oily product was obtained. With excess of succi-

nic anhydride, pure, dry pyridine and only slight heating pure crystals of desoxycholic acid-3-semi-succinate(XLVI) were isolated in good yield. The oxidation to the corresponding 12-keto compound(XLVII) proceeded smoothly and after saponification, methylation and acetylation pure methyl 3-acetoxy-12-keto-cholanate(XLV)was obtained.

Neither of these methods gave yields comparable to those given in the literature, and an analogous new method was, therefore, investigated. The C3-semi-phthallate of desoxycholic acid, prepared with phthallic anhydride and pyridine, was separated from phthallic acid by extraction with chloroform. Oxidation of the C_3-semi-phthallate (L) with chromic acid yielded the corresponding ketone (LI), and on hydrolysis 3-hydroxy-12-keto-cholanic acid (XLVIII) was obtained. This method gave good yields on a small scale run. It was, however, not applied to larger batches.

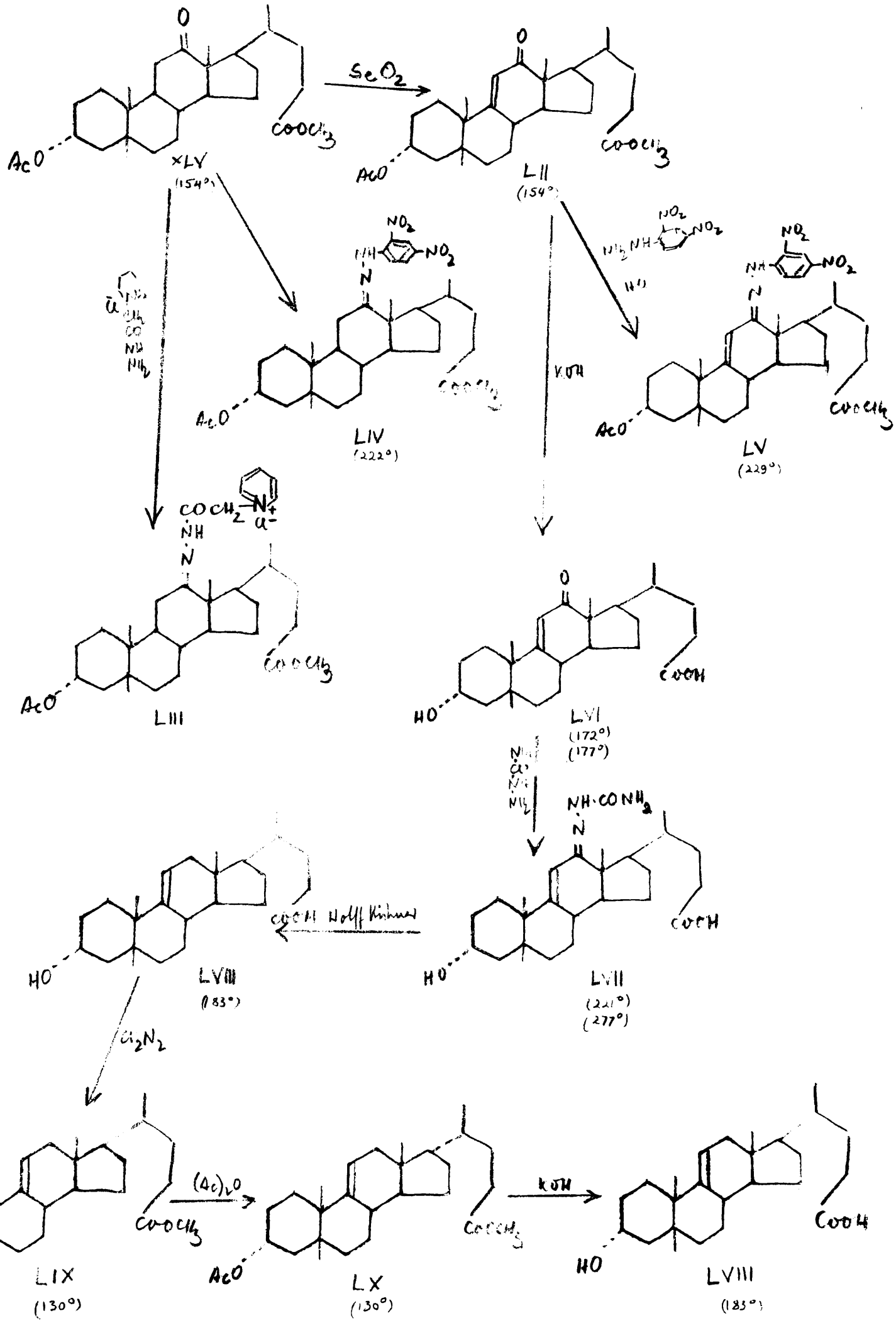
The dehydrogenation of methyl 3-acetoxy-12-keto-cholanate (XLV) with selenium dioxide has been described by Schwenk (420,421), whose directions were followed. Because the dehydrogenation product contains an α,β-unsaturated ketone group, its ultraviolet absorption spectrum shows a maximum at 240 mμ with a molar extinction coefficient (E_m) of 11500. From the E_m of the reaction product it is, therefore, possible to determine the concentration of the unsaturated compound, which does not give a depression of the mixture melting point


XLII
(176°)
XLIII
(80°)
XLIV
(128°)
XLV
(154°)
XLVI
231°
229°
XLVII
(242°)
XLVIII
(160°)
XLIX
(110°)
L
(140°)
LI
(239°)
AcOH
KOH

with the starting material and possesses the same melting point. Thus it was found, that only 50% of the material had been dehydrogenated to methyl 3-acetoxy-12-keto-Δ9:11-cholenate. (LII)

The separation of the 2 compounds by different reactivity with Girard's reagents (191) was studied. Three successive separations were made and 10% of the material was found each time in the reactive("ketonic") fraction. The molar extinction coefficient was observed to increase in the non-reactive ("non-ketonic") fraction and the melting point of the crystals became sharper. In the final "non-ketonic" fraction the E_m reached 11350, while in the "ketonic"fraction it was calculated to be 10550. As a method of separation and purification, however, it appears to be too tedious and expensive.

The reaction with 2,4-dinitro-phenylhydrazine was then investigated. Both compounds form dinitro-phenylhydrazones differing only little in their melting points. When the reaction was studied with 80% pure methyl 3-acetoxy-12-keto-Δ9:11-cholenate, pure crystals of the compound LII hydrazone (LV) separated on standing. The second crop showed a lower and broader melting point. The recovery of the starting material(LII) from its hydrazone(LV) did not succeed. Consequently, the direct Wolff-Kishner reduction of the dinitro-phenylhydrazone was investigated; the desired product could not be isolated.


SeO_2
LV (154°)
LII (154°)
LIV (222°)
LV (229°)
LIII
LVI
(172°)
(177°)
LVIII (183°)
LVII (221°) (277°)
Cl_2N_2
Wolff Kishner
LIX (130°)
$(Ac)_2O$
LX (130°)
KOH
LVIII (185°)

In a second selenium dioxide dehydrogenation the reaction was allowed to continue until completed. After 15 hours the reaction was interrupted, a sample was extracted, and the crystals tested spectrophotometrically. Only 60% had been dehydrogenated. The material was, therefore, subjected to a further dehydrogenation with fresh selenium dioxide for 13 hours. A sample showed a molar extinction coefficient of 10230 and after one more hour, the reaction was terminated and the material was extracted. The crystals had an E_m of 11400, the mother liquors of 10040. The 3-acetoxy-12-keto-Δ9:11-cholenic acid methyl ester (LII) was finally obtained in good yield.

The formation of the semicarbazone of the compound, according to directions given in the literature(430-432), did not succeed. The ester (LII) was saponified and the semicarbazone of the acid (LVII) was then obtained in good yield without difficulties.

Wolff-Kishner reduction of the semicarbazone (LVII) was done with sodium benzylate in benzyl alcohol, as described by Ruzicka (433). The reaction product was precipitated by the addition of saturated sodium chloride solution, methylated, acetylated and chromatographed. Crystals of 3-acetoxy-Δ9:11-cholenic acid methyl ester (LX) were eluted in 57% yield.

To study the nitrogen determination by the semi-micro Kjeldahl method on nitro compounds, 6-nitro-cholesteryl acetate (XXXIII) was prepared. Without preliminary reduction of the nitro group, the amount of ammonia formed accounted for only 70% of the nitrogen present. On reduction with hydrogen iodide the yield of ammonia varied considerably, and on addition of glucose, before digestion, no ammonia could be detected. As the nitro compound is not soluble in hydriodic acid, the reduction was carried out in acetic and hydriodic acid, and the ammonia determinations, accounting for 97% of the nitrogen, could be duplicated. The method was, therefore, thought to be sufficiently reliable to be applied to the nitration products of Δ 9:11-lithocholenic acid.

The first nitration was carried out on methyl 3-acetoxy-12-keto-Δ9:11-cholenate with a mixture of red fuming and concentrated nitric acid. The oily residue could not be crystallized. After reduction with zinc in acetic acid and hydrolysis, the oily residue was chromatographed, and the oils, eluted with benzene and ether, were free of nitrogen, while the ether, chloroform eluates contained some nitrogen.

When using a nitration mixture of fuming and concentrated nitric acid, crystals could be isolated after chromatography. They were not affected by reduction with zinc, but could not be obtained after hydrolysis. The oil was found to be free of nitrogen.

As hydrolysis of the C_3-acetoxy group is likely to happen in the presence of nitric acid, all further nitrations were carried out on 9:11-lithocholenic acid, to eliminate this side-reaction and possible interfering effects of the ester group. A sample was nitrated and the crystalline product, m.p.239-241°C, was analysed by the Kjeldahl method. It was found to have contained one atom of nitrogen per molecule. In the next experiment 9:11-lithocholenic acid was reduced after nitration, and the crystals obtained did not yield any ammonia on analysis. On spectrophotometry, the absence of a nitro group was confirmed.

It was concluded, that on nitration nitrogen enters the molecule, presumably at the C_3-position forming a nitrate, which is removed again during reduction in acetic acid. As the crystals did not show the expected melting point of the starting material, their identification was undertaken, even though hope for the introduction of an oxygen atom at the 11-position by this method had to be abandonned.

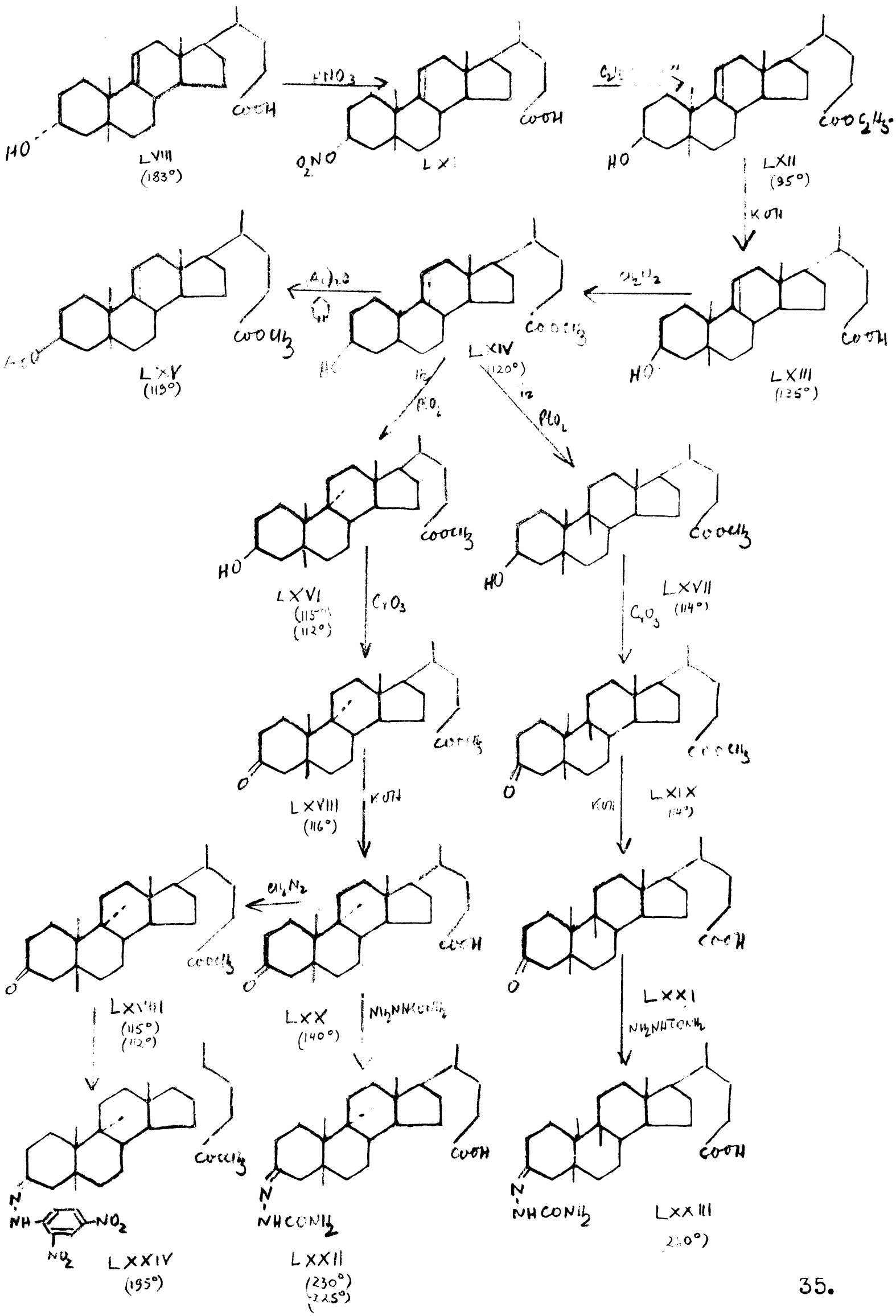
On nitration and hydrolysis in ethanolic hydrochloric acid crystals, M.P. 92-95°C, were isolated (LXII). The crystals were saponified and methylated with diazomethane, yielding a crystalline product, melting at 119-120°C, (LXIV) which could not be identified; neither was the acetylated compound characterized. As all these compounds were unsaturated, the possibility had to be considered, that the double bond had shifted.

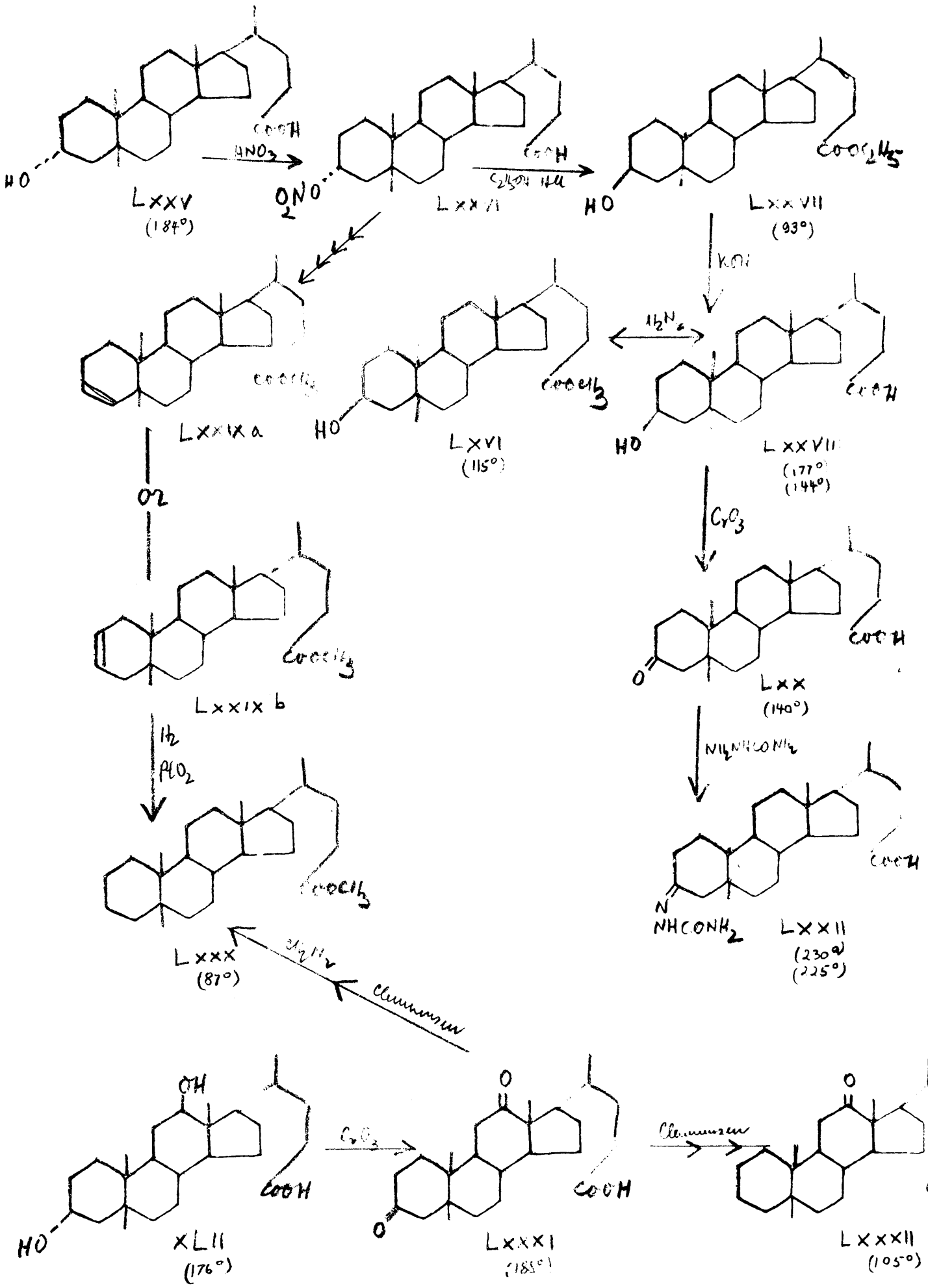
The product was, therefore, reduced with hydrogen in acetic acid and platimum oxide as catalyst. The uptake of hydrogen amounted to 1.05 moles per mole steroid. The product was separated chromatographically into two crystalline compounds, epimeric in the C_9-position. One was identified as belonging into the C_3-epilithocholic acid series, while the other one is a urane derivative. The C_3-ketones were prepared, one of which could be identified. The free acids were transformed into their semicarbazones and the 2,4-dinitro-phenylhydrazone of methyl 3-keto-cholanate was also prepared for further identification.

The presence of a β-hydroxyl at C_3 was substantiated by the formation of insoluble digitonides, which were not formed with any of the bile acids (α-hydroxyl at C_3.)

Further evidence for the assumption that the 9:11 double bond plays no role in these reactions was gained from the nitration and hydrolysis of lithocholic acid. The crystalline nitration product showed a high melting point and showed an orange colour in alkaline solution. After hydrolysis in methanol methyl 3-epilithocholate was isolated. The free acid, the ketone and the semicarbazone were prepared and identified by their melting points. The formulae and melting points of all the compounds described are given on the next page.

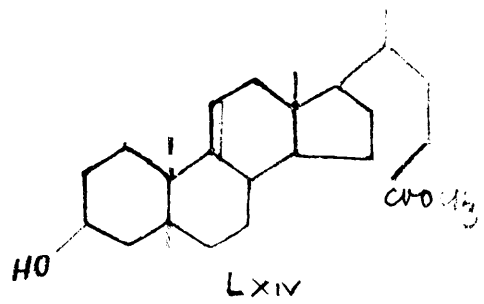
Another crystalline compound had been isolated, after nitration and hydrolysis, in small yield from all chromatograms. The crystals have a low melting point and are very soluble in petrol ether. They were thought to be 2:3 or 3:4-cholenic acid


HO
COOH
LVIII
(183°)
HNO3
O2NO
LXI
C2H5
COOC2H5
HO
LXII
(95°)
KOH
HO
COOH
LXIII
(135°)
LXIV
(120°)
LXV
(119°)
LXVI
(115°)
(112°)
CrO3
LXVII
(114°)
LXVIII
(116°)
KOH
LXIX
(114°)
LXVIII
(115°)
(112°)
LXX
(140°)
LXXI
NH2NHCONH2
LXXII
(230°)
(225°)
LXXIII
(230°)
LXXIV
(195°)
NO2
NH2

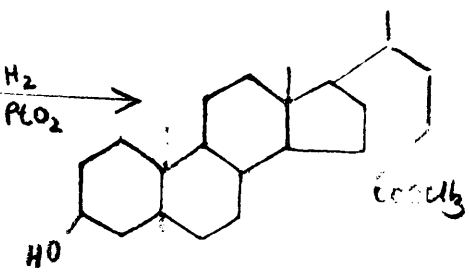

HO
LXXV
(184°)
HNO_3
O_2NO
LXXVI
C_2H_5OH HCl
HO
LXXVII
(93°)
KOH
LXXIXa
LXVI
(115°)
H_2N_2
HO
LXXVIII
(177°)
(144°)
O_2
CrO_3
LXXIX b
H_2
PtO_2
O
LXX
(140°)
$NH_2NHCONH_2$
N
$NHCONH_2$
LXXII
(230°)
(225°)
LXXX
(87°)
H_2N_2
Cleavage
OH
HO
XLII
(176°)
CrO_3
O
O
LXXXI
(185°)
Cleavage
O
LXXXII
(105°)

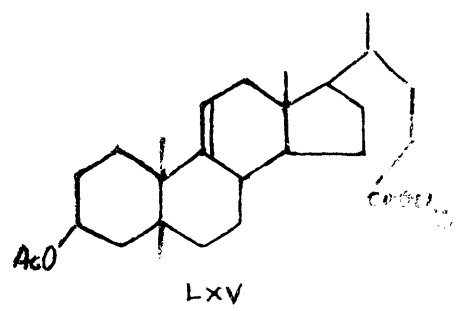
methyl ester, respectively the 2:3 or 3:4,9:11-choladienic acid methyl ester. For identification, the 2:3 or 3:4-cholenic acid methyl ester was reduced with hydrogen and platinum oxide to cholanic acid methyl ester. By Clemmensen reduction of methyl desoxycholate, after oxidation to the diketo compound, methyl cholanate was obtained, which did not give a depression of the melting point when mixed with crystals of the reduction product of methyl 2:3-cholenate.

During hydrogenation experiments with platinum oxide in acetic acid, the surprising observation was made, that the 9:11 double bond could be reduced in epilithocholenates, but not in lithocholenic acid or its ester. A difference in the reactivity of the 9:11 double bond towards nitric acid in lithocholenic acid and its C_3-epimer is conceivable, and may be worth while investigating.

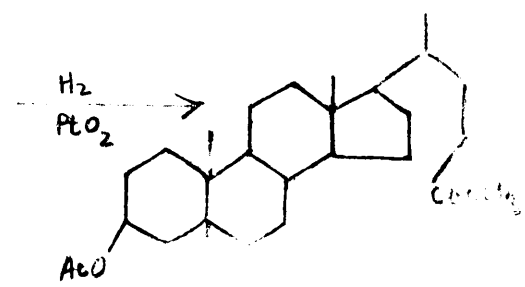


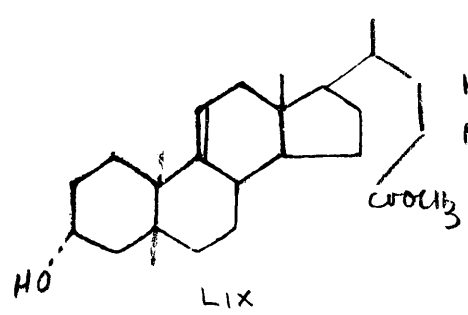
LXIV



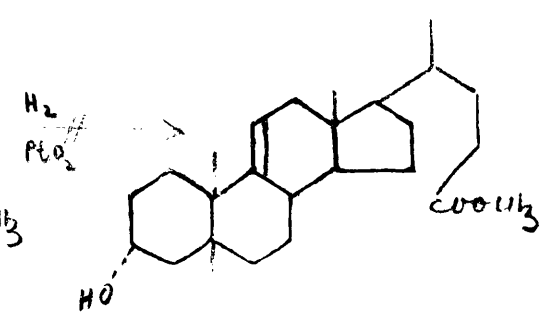


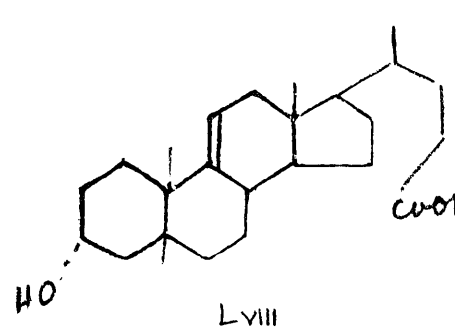
LXV



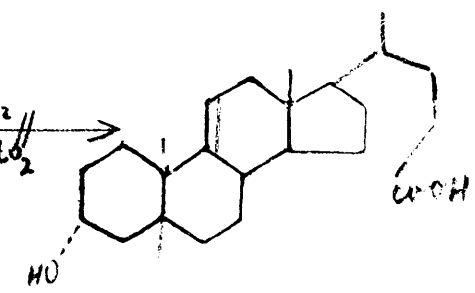


LIX





LVIII



3. Experimental work.

1. Purification of desoxycholic acid (XLII).

Impure desoxycholic acid (100 g.) was purified by methylation and saponification (Reichstein(413)). The crystals were suspended in 150 ml.methanol. Concentrated sulphuric acid (1.5 ml.) was added dropwise with stirring, and the agitation was continued at room temperature, until most of the material had dissolved. The solution was then filtered, and the filtrate crystallized on standing. The crystals were filtered and recrystallized from methanol. m.p.94-111°C.

From the mother liquors on condensation more crystals were obtained, showing the same melting point. The yield was 92.6%. In subsequent methylations the volume of methanol used was reduced to 100 ml.per 100 g. acid (XLII).

Methyl desoxycholate (XLIII)(55.2 g.) was dissolved in methanol(165 ml.), potassium hydroxide (55 g. dissolved in 55 ml.of water) was added, and the mixture was refluxed on the steam bath for 2½ hours. After cooling, the solution was diluted to ten volumes with ice water. On acidification of the clear solution with 6N hydrochloric acid, a white precipitate formed, which was filtered and washed free of acid. The residue was dried in an oven at 80°C, dissolved in methanol and crystallized from acetone. The crystals

of desoxycholic acid (50 g.) showed a double melting point at 160 and 176°C and were, therefore, dried in an oven at 105°C; m.p. 176°C.

2. Preparation of 3-acetoxy-12-keto-cholanic acid methyl ester (XLV) by partial acetylation. (XLII - XLV).

Purified desoxycholic acid (100 g.) was methylated as described above. The crystals were thoroughly dried in a vacuum desiccator; 54 g. of them were dissolved in glacial acetic acid (120 ml.), and concentrated hydrochloric acid (2ml.) was added dropwise. The solution was allowed to stand at room temperature for 24 hours and filtered. The monoacetate (XLIV) was not isolated. Instead, the solution was cooled in an ice bath, and chromic acid (8.03 g. in 10 ml. of water and 30 ml. of acetic acid) was added slowly with shaking. When the solution had turned blue, an additional amount of chromic acid (4 g. in aqueous acetic acid) was added. After standing for 16 hours at room temperature, an excess of chromic acid was still detectable by the test, previously described (page 17). The solution was then diluted to ten volumes with water, and the precipitate was filtered, washed neutral and recrystallized from methanol, m.p. 142-144°C.

Since the melting point of 3-acetoxy-12-keto-cholanic acid methyl ester is given in the literature as 152-154°C, the product was suspected to be contaminated with its products of hydrolysis. The material was, therefore, reacetylated without

however, altering the melting point. On recrystallization from ether, crystals were obtained, having the characteristic melting point of compound XLV, 152-154°C.

3. Preparation of 3-acetoxy-12-keto-cholanic acid methyl ester (XLV) by succinoylation. (XLII, XLVI, XLVII, XLVIII, XLV)

Desoxycholic acid (32.5 g.) was added to a solution of succinic anhydride (84.5 g.) in pyridine (325 ml.) and heated under anhydrous conditions on the steam bath for one hour. The dark solution was then poured into 10 volumes ice water, containing 162.5 ml. concentrated sulphuric acid. A brown, partially crystalline oil separated. Most of the crystals were starting material, m.p. 174-176°C, and some were the semi-succinate, m.p. 211-229°C.

For subsequent succinoylations, the pyridine was refluxed over barium oxide and distilled under anhydrous conditions. On heating the solution of succinic anhydride and pyridine, it turned pink and brown, which was not observed at room temperature. To compensate for lower temperature, excess succinic anhydride (10 equivalents) was used and succinoylation was allowed to continue for 40 hours at room temperature. On extraction, a mixture of starting material and semi-succinate was obtained, from which the latter was separated by fractional crystallisation. 78% yield of semi-succinate, m.p. 236°C was obtained.

Better yields were obtained yet by heating the solution for an hour at 60°C and allowing it to stand at room temperature for 16 hours. A 20-fold dilution of the reaction mixture with acidified ice water was preferred to the 10-fold dilution, described in the literature (415,416), as it prevented the formation of oils and simplified the filtration and washing of the crystals. On recrystallisation from methanol, the first two crops of crystals showed the melting point (227-229°C) of the semi-succinate (66% yield), while later crops indicated the presence of desoxycholic acid besides the semi-succinate. The crystals were again succinoylated.

Oxidation of 3-semisuccinate of desoxycholic acid.

A solution of semisuccinate (XLVI) (21.09 g.) in acetic acid (150 ml.) was cooled in an ice bath. Chromic acid (4 g. in 5 ml. of water) was added dropwise with shaking. Crystals separated from the solution, which were filtered and washed, m.p. 242-243°C. The filtrate did not contain any chromic acid and more (0.5 g.) was therefore added; the solution was then kept at room temperature for 16 hours, diluted with ten volumes ice water and filtered. The residue was washed, dissolved in chloroform, and crystals were isolated on evaporation of the solvent. m.p. 242-244°C.

Saponification of 3-hydroxy-12-keto-cholanic acid-3-semi-succinate. (XLVII).

The reaction product (XLVII) was dissolved in 75 ml. of methanol, and 37.5 ml.6 N sodium hydroxide solution was added. The mixture was refluxed for two hours on the steam bath, diluted to ten volumes with water, acidified and filtered. The product was recrystallized from ether showing a double melting point at 127° and 160°C.

Methylation and Acetylation of 3-hydroxy-12-keto-cholanic acid.

The crystals (XLVIII) were dissolved in ether and methylated with diazomethane (prepared from 1 g.nitrosomethyl-urea) for 15 minutes at room temperature. Excess diazomethane was removed by distillation, with the necessary precautions, as previously described. The residue was not crystallized, but was refluxed for three hours on the water bath with acetic anhydride (15 ml.) and pyridine (15 ml.). After dilution with ten volumes of water, containing enough hydrochloric acid to neutralize the pyridine, crystals separated, which were filtered, washed and recrystallized from ether. m.p.152-154°C.

4. Preparation of 3-acetoxy-12-keto-cholanic acid methyl ester by way of the semi-phthallate.(XLII,L,LI,XLVIII,XLV)

Desoxycholic acid(1 g.) was added to a solution of phthallic anhydride(3.9 g.) in pyridine (20 ml.). After standing for 16 hours at room temperature under anhydrous conditions, the solution was poured into ten volumes of water, containing 12 ml.concentrated sulphuric acid. The white crystalline precipitate was filtered and washed. The majority

of crystals melted at 130-131°C and was identified as phthallic anhydride, while the higher melting crystals, m.p.144-145°C, were assumed to be the desired semi-phthallate. The crystals were treated with 5% sodium carbonate solution (20 ml.), in which they all dissolved(phthallic anhydride is easily hydrolized to phthallic acid), and the solution was acidified. To remove the semi-phthallate of desoxycholic acid from phthallic acid, the precipitate was extracted with chloroform (3x25 ml.), in which phthallic acid is insoluble, followed by ether extraction (3x25 ml.). Both extracts were washed neutral and taken to dryness. From the ether extract crystals of phthallic acid, m.p.200-201°C, were obtained, while the residue from the chloroform extraction was crystallized from ether yielding crystals, m.p.143-145°C, assumed to be the 3-semiphthallate of desoxycholic acid.

Some of the crystals (100 mg.)(L) were saponified with methanolic 2N potassium hydroxide solution (5 ml.) for two hours under reflux. The solution was diluted with ten volumes of water, acidified and filtered. The washed residue was shaken up with chloroform (3x15 ml.), and the chloroform solution was taken to dryness. The crystalline residue was recrystallized from acetone giving the characteristic melting point of desoxycholic acid (m.p.176°C). The chloroform insoluble residue was crystallized from methanol and identified as phthallic acid. Both acids were recovered in equivalent amounts.

Oxidation of desoxycholic acid-3-semi-phthallate (L).

The crystalline semi-phthallate (L)(0.8 g.) was dissolved in acetic acid (12.5 ml.), and chromic acid (120 mg. in 0.5 ml. of water) was added dropwise to the shaken and cooled solution. The mixture was allowed to stand for 5 hours at room temperature, and on cooling, crystals separated from the solution. m.p. 238-239°C. Compound LI.

Saponification.

The keto-semi-phthallate (LI)(435 mg.) was refluxed for three hours in 2N methanolic sodium hydroxide (5 ml.) The solution was diluted with ten volumes of water, acidified and extracted with chloroform (3x25ml). After washing, the chloroform was distilled, and the residue was crystallized from ether showing the double melting point of 3-hydroxy-12-keto-cholanic acid.

On methylation and acetylation, as previously described, methyl 3-acetoxy-12-keto-cholanate(XLV) was obtained. The yields of all reactions were nearly quantitative.

5. Dehydrogenation of 3-acetoxy-12-keto-cholanic acid methyl ester with selenium dioxide. (XLV - LII).

A solution of 11.66 g. compound XLV was dissolved in 45 ml. acetic acid and refluxed in an oil bath at 130-135°C. Selenium dioxide (5.56 g.) was added in small dosages during three hours through the condenser.

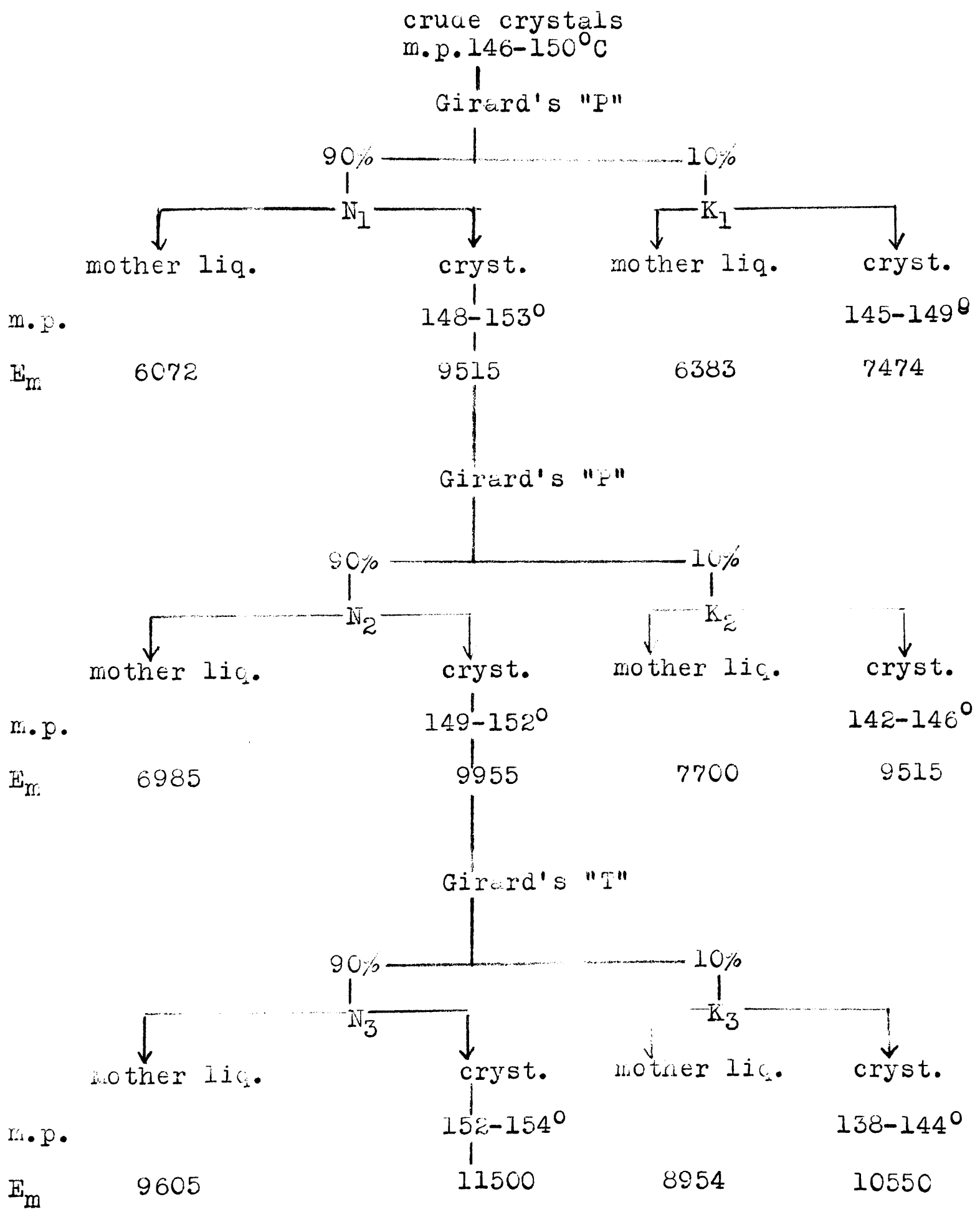
During 12 hours of refluxing, the solution turned from yellow to dark red and a precipitate of selenium settled out, which was removed by filtration. To the filtrate, three volumes of methanol were added, and on ten-fold dilution with ice water a yellow precipitate was obtained, which was extracted with ether (3x400 ml.). The ether was washed with water, dried with sodium sulphate and taken off. The coloured crystals melted at 146-150°C.

A sample of the crystals (cr.10 mg.) was weighed accurately, diluted with ethanol in a volumetric flask (50ml.) and their absorption spectrum was determined. The molar extinction coefficient of compound LII at λ_{max} 240mμ is 11500. From the observed extinction the concentration of compound LII could, therefore, be calculated: 50% compound LII.

6. Purification with Girard's reagents.

To isolate the dehydrogenated product from the starting material, the crystals (11.6 g.) were thoroughly dried in a desiccator, dissolved in absolute ethanol (200 ml.) and refluxed for 1½ hours with Girard's reagent "P"(44 g. (10 equivalents)) and glacial acetic acid (20 ml.) under anhydrous conditions. The cooled solution was then poured into two liters ice water, containing sufficient standard sodium hydroxide solution to neutralize the acetic acid used. The pH was checked and adjusted to 6.8 if necessary. The solution was then extracted with ether(3x600 ml.) The total

Separation of (LII) from (XLV) by Girard's reagent.



ethereal solution was washed with water, which was added to the aqueous phase containing the ketonic complexes, and taken to dryness. From it, 90% of the total material was recovered. The ketonic complexes were hydrolyzed by the addition of concentrated hydrochloric acid to a concentration of 1 N. After two hours at room temperature the liberated ketones were extracted with ether(3x600 ml.) The ether was washed and distilled. Ten percent of the material was recovered from this fraction, reacting with Girard's reagent("K"). From the measurement of the ultraviolet absorption spectrum, the purity of compound LII in the reactive("K") and in the non-reactive ("N") fraction could be calculated. The separation was repeated once more with Girard's reagent "P" and once with reagent "T", giving the same distribution of 90% non-reactive and 10% reacting with Girard's reagents. The purification was followed by measurement of E_m and the melting point of the fractions, and the results are condensed in the table on page 47.

7. Formation of the 2,4-dinitro-phenylhydrazones (LIV,LV)

Pure crystals of compound LII (1 g.) were dissolved in methanol (20 ml.) and added to a solution of 4.55 g. of 2,4-dinitro-phenylhydrazine in methanol (200 ml.). After the addition of two ml. of concentrated hydrochloric acid, the solution was allowed to stand at room temperature for 16 hours. On standing in the refrigerator, needles separated, which were filtered and washed with dilute hydrochloric acid and water.

The crystals melted at 226-229°C. The filtrate was condensed to a small volume, diluted ten times with water and extracted with ether and chloroform. Crystals of dinitro-phenylhydrazine were isolated, m.p.194-201°C with decomposition, which show a colour change from red in neutral to yellow in acid and blue in alkaline solution, while the dinitro-phenylhydrazones, prepared in this investigation, do not show this reaction. Use was made of this discovery in the distinction of the products.

The 2,4-dinitro-phenylhydrazone of pure 3-acetoxy-12-keto-cholanic acid methyl ester (LIV) was prepared in the same way. The yellow needles melted at 219-222°C.

The preparation of the 2,4-dinitro-phenylhydrazone from an impure sample of the dehydrogenation product (70% pure) gave rise to pure 3-acetoxy-12-dinitro-phenylhydrazone-Δ9:11-cholenic acid methyl ester (LV). The filtered solution was condensed to ½ volume, and another crop of crystals was obtained, m.p.190-195°C, presumably a mixture of the two dinitro-phenylhydrazones.

Hydrolysis and Wolff-Kishner reduction of 2,4-dinitro-phenylhydrazone. (LV).

A suspension of 100 mg. compound LV in methanol (50 ml.) and hydrochloric acid (10 ml. 6N) was refluxed for 24 hours. The solution was then filtered and diluted with ten volumes of water. A crystalline precipitate was obtained, which, however, was not the desired product (LII).

The Wolff-Kishner reduction was carried out on 275 mg. compound LV, as described in detail in section 10. On addition of the crystals to the solution of sodium benzylate in benzyl alcohol, an immediate blackening was observed. After refluxing for four hours on an oil bath at 180-190°C the black colour had disappeared. On extraction a brown oil was obtained which could not be crystallized.

5A. Improved dehydrogenation of 3-acetoxy-12-keto-cholanic acid methyl ester (XLV) with selenium dioxide.

The crystals (XLV) (28 g.) were dissolved in acetic acid (110 ml.), and the air in the reaction flask was displaced by a current of dry nitrogen. The solution was then refluxed under nitrogen at 115-125°C on the oil bath, and selenium dioxide (14 g.) was added in small amounts over two hours. After 15 hours, a sample of the red-brown solution was extracted, as described in section 5, page 46, and its molar extinction coefficient was determined. Only 60% of the material had reacted.

The sample was returned to the solution and the dehydrogenation was continued for 13 hours with a new batch of selenium dioxide (15 g.). A five ml. sample of the solution was withdrawn, extracted and tested spectrophotometrically. The molar extinction coefficient amounted to 10230. After another hour, the reaction was stopped. Selenium and its unreacted dioxide were filtered off and chromic acid (400 mg.) was added,

to destroy organic selenium compounds. The solution was diluted with ten volumes of water, and the precipitate was filtered, dissolved in hot ether (200ml.), washed with 5% sulphuric acid (3x60 ml.), with 5% ice cold sodium bicarbonate solution (3x60 ml.), with water until neutral. The ether was then dried with sodium sulphate and evaporated. The product crystallized from ether, m.p. 152-154°C, E_m 11400. From the mother liquors crystals with E_m 10010 were obtained.

8. Saponification of 3-acetoxy-12-keto-Δ9:11-cholenic acid methyl ester. (LII).

As the semicarbazone formation of compound LII had not succeeded, the crystals (LII)(20 g.) were dissolved in 120 ml. of methanol and 60 ml. 6 N sodium hydroxide solution and refluxed for 16 hours on the steam bath. The solution was cooled, poured into ten volumes of ice water, giving a clear solution, and acidified. The yellow precipitate was filtered, washed with water and crystallized, m.p. 177°C. (LVI)

9. Semicarbazone formation of 3-hydroxy-12-keto-Δ9:11-cholenic acid. (LVI).

The acid (LVI)(2.971 g.) was dissolved in a mixture of ethanol(30 ml.) and dry pyridine(30 ml.), and the semicarbazide solution (3 g. semicarbazide hydrochloride in 8 ml. of water and 3 g. sodium acetate in 40 ml. ethanol, mixed and filtered) was added, and the mixture refluxed for two hours

on the steam bath. The solution was cooled, poured into acidified ice water (600 ml.), and the crystalline precipitate was filtered and washed. The crystals (LVII) showed a sharp melting point at 277°C, on recrystallization from methanol. The yield was 2.693 g. The reaction was repeated a number of times, always giving good yields of the semicarbazone, suggesting, that steric hindrance must have prevented the reaction in the case of the corresponding methyl ester.

10. Wolff-Kishner reduction of the semicarbazone (LVII).

Pure sodium metal(2.9 g.) was quickly dried on a filterpaper, cut into small pieces and dropped into benzyl alcohol(35 ml., dried over Drierite and freshly distilled). After an initial vigorous reaction, the dissolution of sodium slowed down, and the flask was heated on the steam bath under vacuum, to remove toluene and speed up the evolution of hydrogen. After 7 hours the sodium was dissolved and the solution solidified on cooling.

The semicarbazone(3 g.)(LVII) was added to the sodium benzylate, and the mixture was refluxed under a stream of dry nitrogen for 4 hours at 180-190°C. The solution was then steam distilled for ½ hours, until a clear distillate was obtained, which was discarded. The brown solution was treated with an equal volume saturated sodium chloride solution, which caused the precipitation of an oily material. The oil was separated by centrifugation; the supernatant solution was decanted

and acidified.Pure crystals of benzoic acid, m.p.125-127°C, were isolated. The oily residue was extracted with acidified ether (60 ml.), which was washed with N sodium hydroxide solution(3x20) (3x20 ml.). The aqueous extracts were acidified and reextracted with ether (3x25 ml.)The ethereal solution was washed neutral, dried and taken to dryness.The residue was an oil.(2.7 g.)

11. Methylation and acetylation of Wolff-Kishner products.

The oily residue(2.7 g.) was dissolved in dry ether and methylated with diazomethane, as previously described.On extraction, an oil was obtained, which could not be crystallized. Acetic anhydride (3 ml.) and dry pyridine (9 ml.) were added to the oil, and the solution was allowed to stand at room temperature for 16 hours under anhydrous conditions.The solution was diluted with ten volumes acidified water, and the precipitate was extracted with ether (3x40 ml.) The ether was washed with iced 5% sodium carbonate solution (3x20 ml.), with water until neutral, dried with sodium sulphate and evaporated. The residue crystallized from methanol.The crystals (1.673 g.) melted at 124-131°C.

12. Chromatography of crystalline product of section 11.

The crystals(3.83 g.) were dissolved in dry benzene, (5 ml.) and petrol ether was added to incipient cloudiness.The solution was then poured on a column of alumina (120 g., Harshaw, acid washed, reactivated) which had been washed with petrol ether. The chromatogram is given on page 54.

Chromatogram of crystalline products of section 11.

Eluant 50 ml.		mg. Wt.	Eluate nature	m.p.	formula	tetranitro methane
1. P.E.:	Bz (9:1)	1.1	oil			
2. "	"	3.4	"			-
3. "	(4:1)	0.6	"			-
4. "	"	8.1	"			-
5. "	(2:1)	132.0	cryst.	134-136°	LX	-
6. "	(1:1)	366.6	"	"	"	+++
7. "	"	664.9	"	"	"	+++
8. "	"	538.5	"	"	"	+++
9. "	"	245.0	"	144-152°	LII	+++
10. "	"	124.3	"	152, 177°	?	+
11. "	"	74.0	"	"	?	+
12. "	"	90.2	"	"	?	+
13. "	"	115.5	"	170-176°	?	+
14. "	"	46.5	"	134-162°	?	+
15. "	"	51.8	"	170-174°	?	+
16. "	"	76.3	"	"	?	+
17. "	"	75.1	"	166-170°	?	+
18. Bz	100%	24.0	"	147-164°	?	+
19. "	"	111.8	oil			+
20. "	"	119.6	"			+
21. "	"	86.7	"			+
22. Bz.:	Eth. (9:1)	57.4	"			+
23. "	"	82.6	"			+
24. "	(4:1)	163.1	"			+
25. "	"	91.7	"			+
26. "	(2:1)	74.3	"			+
27. "	(1:1)	127.1	"			+
28. "	"	129.1	"			
29. Ether	100%	80.3	"			
30. $CHCl_3$	"	13.8	"			
31. CH_3OH	"	1.7	"			
Total eluted		3776.1	"			
total added		3830.	"			

13. Nitration of cholesteryl acetate.(XXXII).

Cholesteryl acetate, m.p.101°C, (5 g.) was suspended in acetic acid (30 ml.) and a nitric acid mixture (30 drops) (20 ml.concentrated nitric acid, spec.grav.1.5, and 12.5 ml. fuming nitric acid, spéc.grav.1.515) was added with stirring. The solution was cooled in a freezing mixture and the rest of the nitric acid solution was added dropwise from a burette to the agitated solution. The solution was stirred for one hour, before it was poured into 400 ml. of water. The white precipitate was filtered, washed neutral with water and recrystallized from methanol. m.p.102-104°C.

14. Semimicro-Kjeldahl nitrogen determination on 6-nitro-cholesteryl acetate.(XXXIII).

Samples of the nitro compound (XXXIII)(cr.35 mg.) were weighed accurately on a cigarette paper (without the adhesive) and dropped with the paper into a small round-bottomed, long-necked, standard taper flask. The digestion mixture, used in all experiments, consisted of concentrated sulphuric acid (3 ml.), copper sulphate, selenium dioxide (a few mg. each) and potassium sulphate (approx. 1 g.). Two boiling stones were added to the mixture to prevent bumping.

In series A, digestion was carried out on the nitro compound without previous reduction. In series B, the compound was first reduced by boiling with hydrogen iodide

(1 ml.) and red phosphorus(3 mg.) for 30 minutes. The neck of the flask was then sprayed with water, and concentrated sulphuric acid (2 ml.) was added, and the solution heated till the water, hydrogen iodide and iodine had been driven off. The solution was then digested as in series A. Digestion of the nitro compound in series C was carried out as in A, except for the addition of 100 mg. glucose before heating. All three methods were found unsatisfactory. As the compound was insoluble in hydrogen iodide, the preliminary reduction of the nitro compound was carried out in acetic acid giving reproducible results. (series D.)

The percentage of nitrogen in compound XXXIII.			
Series A	Series B	Series C	Series D
2.256%	2.371%	0%	2.810%
2.381%	1.068%	0%	2.808%
2.223%	1.175%		2.789%
2.243%	1.535%		
recovery 70%	recovery 35-70%	recovery 0%	recovery 92%

15. Nitration of 3-acetoxy-Δ9:11-cholenic acid methyl ester. (LX).

The ester (LX)(670.5 mg.) was dried in vacuo and dissolved in glacial acetic acid(2.7 ml.). A few drops of the nitration mixture (2.4 ml. concentrated nitric acid and 1.9 ml. red fuming nitric acid) were added. The solution was cooled in a freezing mixture, and the nitric acid mixture was added dropwise with shaking. After standing for 90 minutes at room temperature, the solution was poured into ice water (200 ml.) and

extracted with ether (3x60 ml.). The ether was washed with water, dried with sodium sulphate and distilled at room temperature. The yellow oil could not be crystallized.

16. Reduction of the nitration product.

Zinc dust(1.33 g.) and 10 ml. acetic acid with 2 ml. of water were added to the oily residue, and the mixture was heated on the steam bath for two hours. Crystals of zinc acetate hydrate, m.p.240°C, formed. The mixture was then refluxed for ten hours under a stream of nitrogen, cooled, diluted with ice water (150 ml.) and extracted with ether, (3x50 ml.). The ether was washed, dried and distilled, leaving an oily residue, which could not be crystallized.

17. Hydrolysis of reduction product.

The residue was refluxed in ethanol(7 ml.) and concentrated hydrochloric acid (2ml.) for 90 minutes. After standing over night at room temperature, the solution was diluted with water (100 ml.) and extracted with ether(3x30 ml.) The ethereal solution was extracted with cold 5% sodium carbonate solution (3x30 ml.), which was acidified and reextracted with ether. The ether was washed, dried and distilled, leaving 57.3 mg. oil. After methylation with diazomethane and chromatography, no crystalline compounds were isolated. The ether solution, containing the neutral material was washed free of sodium carbonate, dried and taken to dryness. The oily residue (445.2 mg.) was chromatographed.

Most of the 34 oily fractions eluted did not contain any nitrogen, while the ether, chloroform eluate contained 1/3 atom nitrogen per mole.

18. Nitration No. 2 of 3-acetoxy-Δ9:11-cholenic acid methyl ester. (LX).

The nitration mixture of this and subsequent nitrations was made up of 5 parts concentrated nitric acid and 4 parts fuming nitric acid. The ester (LX) (50.6 mg.), in acetic acid (1 ml.), was nitrated with the mixture (0.45 ml.) as described above. The oily residue was crystallized from petrol ether, m.p. 98-102 and 111-117°C. After reduction with zinc in acetic acid, an oil was obtained which crystallized from methanol, m.p. 98-105°C. After acid hydrolysis crystals could no longer be obtained, and the oil was found to be free of nitrogen.

19. Saponification of methyl 3-acetoxy-Δ9:11-cholenate. (LX).

The ester (LX) (525 mg.) was dissolved in methanol (4 ml.) and 6 N sodium hydroxide (2 ml.) and refluxed on the steambath for two hours. The solution was poured into ten volumes of water, washed with ether (3x20 ml.), acidified and extracted with ether (3x40 ml.) The ether was washed, dried and distilled. Crystals of Δ9:11-lithocholenic acid, m.p. 188°C were obtained.

20. Methylation and acetylation of Δ9:11-lithocholenic acid.

The acid(LVIII)(306 mg.) was suspended in ether (10 ml.) and treated with diazomethane. When the crystals had dissolved and the solution remained yellow, the excess diazomethane and the solvent were distilled. The residue crystallized in rosettes, m.p. 108-109°C; a sample was retained for mixture melting point determination with the nitration product.

The remaining crystals(LIX) were dissolved in acetic anhydride(2.4 ml.) and dry pyridine(6 ml.) and left for 40 hours at room temperature under anhydrous conditions. The solution was diluted with ten volumes acidified ice water. The crystalline precipitate was filtered and washed with water. m.p. 138-140°C. The yield was quantitative.

21. Nitration of Δ9:11-lithocholenic acid.(LVIII).

The acid(LVIII)(54.3 mg.) was dissolved in acetic acid(1.5 ml.) and nitrated as described before. Needle shaped crystals were obtained from the residue on recrystallization from methanol, which darkened at 236°C and melted at 241°C. A Kjeldahl nitrogen determination on the crystals showed them to contain one atom nitrogen per molecule.

22. Nitration No.2 of Δ9:11-lithocholenic acid(LVIII).

The nitration was repeated on 69 mg. lithocholenic

acid,and the residue was reduced with zinc dust(140 mg.) in acetic acid(3 ml.) The mixture was heated under a stream of nitrogen for two hours on the steam bath, filtered,and the filtrate was diluted with ether(50 ml.) and washed neutral with water,dried with sodium sulphate and taken to dryness. The oily residue(59.7 mg.) crystallized partly(m.p. 104°C) and was sublimed. The sublimate did not crystalize and was found to be free of nitrogen by the sodium fusion test.

The test was first carried out on 6-nitro-cholesteryl acetate. A few crystals and a small piece of sodium were placed into a soft glass micro test tube,heated to red heat and dropped into a 10 ml. beaker,containing distilled water. The contents of the cracked test tube were broken up with a glass rod and filtered. The filtrate was then tested for the presence of cyanide with ferric sulphate.

In the control test a green colour was observed followed by the precipitation of blue crystals while the reduced nitration product gave a negative reaction. These results suggest that the nitrogen containing compound was the 3-nitric acid ester of lithocholenic acid,which was split off on hydrolysis with acetic acid.

23. Nitration No.3 of Δ9:11-lithocholenic acid.(LVIII).

The nitration,reduction and hydrolysis of lithocholenic acid(200 mg.) was repeated. Nearly all the material

was recovered in the neutral fraction. Part of the material crystallized, m.p. 75-90°C, and was purified by chromatography. Of 120 mg., 57 mg. crystals were eluted (petrol ether: benzene 8:2 - 1:1), which melted at 92-95°C. On spectrophotometric analysis they were found to contain neither a nitro nor nitroso group in the molecule.

24. Saponification of ethyl epilithocholenate. (LXII).

The ethyl ester (LXII) (57 mg.) was refluxed with 2 N methanolic sodium hydroxide solution (3 ml.). After 90 minutes reflux, the solution was cooled, diluted with ten volumes ice water, and the clear solution was acidified and extracted with ether (3x25 ml.). The ether was washed, dried and distilled, leaving an oily residue, which crystallized from ether, petrol ether, m.p. 133-135°C.

25. Methylation and acetylation of Δ9:11-epilithocholenic acid. (LXIII).

The acid (LXIII) (50 mg.) was methylated with diazomethane in ether. After standing for 15 minutes in the fume cupboard, the excess diazomethane was destroyed by addition of a few drops of acetic acid, until the yellow colour was discharged. The solution was washed with water, dried and taken to dryness. Crystals were obtained, m.p. 117-119°C, which were chromatographed. With petrol ether: benzene 9:1 to 1:1 crystals were eluted, which melted at 119-120° C.

The ester(LXIV)(40 mg.) was dissolved in dry pyridine (1 ml.) and acetic anhydride(0.2 ml.), and allowed to stand at room temperature for 16 hours under anhydrous conditions. The solution was diluted with ten volumes acidified ice water, extracted with ether (3x10 ml.), and the ether extract was washed and taken to dryness. The crystals melted at 117-119°C.

26. Hydrogenation of methyl 3-acetoxy- 9:11-C_5epi-cholenate. (LXV).

The ester (LXV)(31.4 mg.) was reduced with hydrogen, and platinum oxide(6 mg.) as catalyst, in acetic acid as described in detail in section 28. During two hours shaking in the microhydrogenation apparatus, 115% of the theoretical amount of hydrogen, to saturate one double bond, was taken up. From the solution, crystals were obtained, which were purified by chromatographic separation. With petrol ether: benzene (7:3) crystals were eluted, which were identified as methyl 3-acetoxy-epicholanate.

27. Nitration No.4 of Δ 9:11-lithocholenic acid (LVIII).

The acid(LVIII)(100 mg.)was nitrated in the usual manner. Reduction with zinc was omitted and the product was hydrolyzed with methanolic hydrochloric acid (0.6 ml. in 2 ml. methanol) under reflux for four hours. After cooling, the solution was diluted with ten volumes water, extracted with ether and separated with 10% sodium carbonate solution

into acid and neutral fractions. From the small acid fraction crystals were obtained which melted at 280-290°C.

The neutral fraction contained most of the material, which could not be crystallized. On chromatography crystals were eluted(petrol ether:benzene(4:1to 1:1)), which melted at 118-120°C.methyl 3-epi-Δ9:11-lithocholenate.(LXIV).

28. Hydrogenation of methyl 3-epi-Δ9:11-lithocholenate.(LXIV).

Platinum oxide (6 mg.) and acetic acid (0.5 ml.) were placed into the micro-hydrogenation flask. The crystals (LXIV)(30 mg.) were placed into a small cup,which was suspended from a turnable hook in the side arm. The system was evacuated and filled with hydrogen(generated by the action of zinc on hydrochloric acid and purified by passing through silver nitrate,potassium hydroxide and potassium permanganate solutions) repeatedly to remove all air.The connection was then shunted to a 5 ml.hydrogen reservoir and the flask was shaken mechanically,until the platinum oxide was saturated with hydrogen.The reservoir was refilled, note was taken of the hydrogen level at atmospheric pressure,the time,the temperature and the barometer reading.The cup, containing the ester(LXIV) was dropped to the bottom of the flask,allowing the acetic acid to come in contact with it.The flask was shaken uniformly and the adjusted hydrogen level was read at constant intervals. After 108 minutes the up-take of hydrogen had become very slow, and the reaction was considered completed.

The flask was disconnected, and the solution was filtered recovering the platinum. The hydrogenation apparatus was set up again and shaken for 108 minutes, to measure the leakage of hydrogen in the system. The actual readings are reproduced on the accompanying table.

time	ml. hydrogen gas		$21^{o}C$ 760 mm.	
11.52 a.m.	3.60			
12.00 p.m.	2.45			
12.05 "	1.72		total used	4.6 ml.
12.10 "	1.13			
12.15 "	0.58		total leakage	2.56 "
12.20 "	0.36			
12.25 "	0.16		Actual uptake	2.04 "
12.30 "	0.12			
12.35 "	0.02	1.44	corrected for temperature and pressure	1.859"
12.40 "		1.35		
12.45 "		1.28	theoretical uptake	
12.50 "		1.19	one mole	1.735"
12.55 "		1.10		
1.10 "		0.95		
1.20 "		0.80	106% of theory	
1.30 "		0.67		
1.40 "		0.45	one double bond has	
1.50 "		0.42	been reduced.	
2.00 "		0.40		

The filtered solution, containing the reduction product, was diluted with ten volumes water and extracted with ether (3x30 ml.). The ether was washed, dried and taken to dryness. The residue was chromatographed and crystals were eluted, as recorded (Chromatogram 14.).

Chromatogram 14.

1.	Pet.Eth.	100%	10 ml.	oil		
2.	" :	Bz.9:1	"	crystals	52-54°C	?
3.	"	" 8:2	"	oil		
4.	"	" 7:3	"	crystals	109-114°C	LXVII
5.	"	" 6:4	"	"	"	"
6.	"	" 5:5	"	"	98-103°C	LXVII & LXVI
7.	"	" 4:6	"	"	111-113°C	LXVI
8.	Bz.	100%	"	"	"	"
9.	Bz : Eth.	9:1	"	"	"	"
10.	" "	8:2	"	oil		

A mixture melting point of the crystals in fraction 4 and 7 gave a depression similar to the one observed in fraction 6.Fraction 6 was, therefore, assumed to be a mixture of the two crystalline substances epimeric at C_9-position.

29. Oxidation of methyl C_3-epilithocholate-C_9-epimers.

The crystals of fraction 4 (LXVII) were dissolved in acetic acid(1 ml.) and oxidized with chromic acid (6 mg.) at 0°C. After standing on ice for one hour and at room temperature for another hour, the solution was diluted with ten ml. of water.On standing, crystals formed which were filtered. The filtrate was extracted with ether, which was washed and distilled.Both crops of crystals showed the same melting point, 112-113.5°C.Crystals of fraction 8 of chromatogram 14 were similarly oxidized and crystals were obtained, m.p.116°C, which could be identified as methyl 3-keto-cholanate(LXVIII).

30. Saponification of methyl 3-keto-cholanate C_9-epimers.

The crystals of compound LXVIII and LXIX were separately dissolved in methanol (1 ml.) and 6 N sodium hydroxide solution (0.5 ml.) and refluxed for one hour. The solutions were cooled, diluted with 20 volumes of water, washed with ether, acidified and extracted with ether. (3x20 ml.) The washed ether was dried and distilled. The oily residues could not be crystallized.

31. Semicarbazone formation of compounds LXX and LXXI.

Portions of the oily residues were treated with semicarbazide hydrochloride and sodium acetate as described in detail in section 9. Crystals differing in shape and melting point were obtained from the reactions. The semicarbazone of methyl-3-keto-cholanate (LXXII) melted at 225°C with decomposition, while the semicarbazone of the C_9 epimer melted at 260°C with decomposition.

32. Dinitro-phenylhydrazone formation of methyl 3-keto-cholanate. (LXVIII).

The remaining oily fraction of 3-keto-cholanic acid was methylated with diazomethane and chromatographed. The methyl ester was eluted (petrol ether: benzene 1:1), m.p. 116°C and treated with 2,4-dinitro-phenylhydrazine as described in section 7. On cooling of the solution, crystals of the dinitro-phenylhydrazone were separated. m.p. 193-195°C.

33. Application of the digitonin test to C_3 α-and β-hydroxyl compounds of the lithocholic acid series.

To check the digitonin test, a few mg. cholesterol were dissolved in one drop ethanol in a micro test tube. To the solution 3 micro drops of alcoholic digitonin solution were added, and the precipitation of the insoluble cholesteryl digitonide was observed after a few seconds. The test was then applied to a number of products of nitration and hydrolysis of lithocholic and 9:11-lithocholenic acid as well as to lithocholic and 9:11-lithocholenic acid. The results are recorded below.

lithocholic acid	no precipitate	α	-hydroxyl
9:11-lithocholenic acid	no precipitate	α	"
compound LXII	precipitate	β	"
compound LXIV	precipitate	β	"
compound LXVI	precipitate	β	"
compound LXXVIII	precipitate	β	"

34. Methylation of 3-acetoxy-cholanic acid.

The acid(200 mg.) was suspended in ether and methylated with diazomethane. Not all the material dissolved on standing in the presence of diazomethane at room temperature. The excess diazomethane was distilled, and the colourless solution was filtered, to remove impurities. The filtrate was washed with 5% sodium carbonate solution and with water, dried and taken to dryness. Crystals remained. m.p. 129-131°C.

35. Saponification of methyl 3-acetoxy-cholanate.

The lithocholate(200 mg.) was refluxed for 90 minutes in a solution of methanol(2 ml.) and 6 N potassium hydroxide (1 ml.) The solution was cooled, diluted with ten volumes of water, washed with ether, acidified and extracted with ether(3x25 ml.) The ethereal extract was washed with water, dried and taken to dryness. Crystals, m.p.185-187°C, were obtained.(LXXV).

36. Nitration of lithocholic acid (LXXV).

Lithocholic acid(175 mg.) was nitrated and the oily residue was hydrolyzed with ethanolic hydrochloric acid as previously described. The ether extract was divided into an acidic and neutral fraction by washing with cold 5% sodium carbonate solution.

From the acidic fraction crystals were obtained from ether, petrol ether, which melted at 225-227°C with decomposition. The crystals did not dissolve in ether and could not be methylated with diazomethane. They dissolve readily in aqueous sodium hydroxide solution (1N) giving a deep orange solution. On acidification, colourless crystals were again formed, which melted at 228-230°C on recrystallisation form methanol. They were assumed to be the 3-nitric acid ester of ethyl lithocholate.(LXXVI).

The neutral fraction was chromatographed, and crystals were eluted with petrol ether: benzene 9:1 to 3:2 which melted from 81-90°C. The crystals were washed with petrol ether, giving a sharp melting point at 92-93°C. (LXXVII). The petrol ether washings were retained.

37. Saponification of ethyl epilithocholate. (LXXVII).

The ester (LXXVII) (40 mg.) was saponified by refluxing in a solution of 2 N methanolic potassium hydroxide (3 ml.); the cooled solution was diluted with 30 ml. of water, washed with ether, acidified and extracted with ether. From the washed (neutral) and dried ether, an oil was obtained which did not crystallize.

38. Methylation of epilithocholic acid (LXXVIII).

The oily residue (LXXVIII) was dissolved in ether and methylated with diazomethane. The crystals were purified by chromatography. They were eluted with petrol ether: benzene 4:1 to 1:1 and, after washing with petrol ether, melted at 117-119°C. The petrol ether washings were combined and taken to dryness. The crystals melted at 87-90°C.

39. Saponification of methyl epilithocholate. (LXVI).

The methyl ester (LXVI) (15 mg.) was saponified, as described in section 37, and crystals were obtained which showed a double melting point, 127° and 142-144°C. The melting point does not agree with that given in the literature (177°C).

40. Oxidation of epilithocholic acid.(LXXVIII).

The crystals(LXXVIII)(12 mg.) were dissolved in acetic acid (1 ml.) and oxidized with chromic acid(6 mg.) at 0°C.After standing at room temperature for an hour, the solution was diluted with 20 ml. of water and extracted with ether,(3x20 ml.).The ether was washed,dried and distilled. Crystals of 3-keto-cholanic acid (LXX),m.p.138-140°C, were isolated.

41. Semicarbazone formation of 3-keto-cholanic acid.(LXX).

The semicarbazone of 3-keto-cholanic acid was prepared from semicarbazide hydrochloride,sodium acetate and pyridine as described in section 9. The gelatinized solution was extracted with ether(3x15 ml.),washed with dilute hydrochloric acid, with water until neutral, dried with sodium sulphate and taken to dryness.Small rosettes of crystals formed, which melted at 225°C.(LXXII).

42. Hydrogenation of methyl 2:3 or 3:4-cholenate.(LXXIX).

The crystalline material,obtained from the petrol ether washings in section 38,(LXXIX)(12.6 mg.),m.p.87-90°C, were dissolved in acetic acid (0.5 ml.) and reduced with platinum oxide as catalyst.(The procedure is described in detail in section 28.) After the hydrogen uptake had ceased, the solution was filtered and the platinum was recovered.

The filtrate was diluted to ten volumes with water and extracted with ether(3x15 ml.)The ether extracts were washed neutral with water,dried with sodium sulphate and taken to dryness. The residue was chromatographed,and the fractions eluted were recrystallized.The results are condensed below.

Chromatogram 16.

1.	Pet.Eth.:	Bz. 10:0	10 ml.	crystals 76-80°	LXXX
2.	"	"	"	"	"
3.	"	9:1	"	"	"
4.	"	8:2	"	"	"
5.	"	7:3	"	oil	
6.	"	6:4	"	crystals 112-114°C	LXVI
7.	"	5:5	"	"	"
8.	Bz. 100%		"	"	"
9.	Bz.: Eth.	9:1	"	"	"
10.	"	8:2	"	oil	

The crystals of fraction 1 to 4 were sublimed, m.p.86-87°C. They were assumed to be methyl cholanate.The other crystalline compound was identified as methyl epilithocholate(LXVI).

43. Oxidation of desoxycholic acid.(XLII).

Desoxycholic acid(524.6 mg.) was dissolved in acetic acid(100 ml.) and chromic acid(272 mg. in 4 ml. aqueous acetic acid) was added to the cooled solution dropwise with shaking.After standing for two hours at room temperature chromic acid was still present in the solution, which was diluted with water and extracted with ether.From the washed and dried ether solution, crystals were obtained on evaporation of the solvent.m.p.188-189°C.(LXXXI).

44. Clemmensen reauction of 3,12-diketo-cholanic acid. (LXXXI).

Zinc wool (5 g.) was treated with a 5% aqueous mercurous chloride solution(10 ml.) and shaken repeatedly, while standing at room temperature for one hour. The solution was decanted, and the zinc amalgam was washed once with water. It was then added to a solution of 3,12-diketo-cholanic acid (LXXXI)(500 mg.) in acetic acid(7.5 ml.) and concentrated hydrochloric acid(17.5 ml.), and the mixture was refluxed for three hours with addition of concentrated hydrochloric acid at hourly intervals. The solution was diluted with 100 ml. of water and extracted with ether(3x50 ml.)The ether extracts were washed, dried and taken to dryness. The crystalline residue was methylated with diazomethane. On evaporation of the solvent an oil remained, which was chromatographed, as recorded below.

Chromatogram 17.

1.	Pet.Eth.:	Bz. 10:0	10 ml.	crystals	76-80°C	LXXX
2.	"	"	"	"	"	"
3.	"	9:1	"	"	"	"
4.	"	8:2	"	"	"	"
5.	"	"	"	"	"	"
6.	"	7:3	"	"	103-105°C	LXXXII
7.	"	"	"	"	"	"
8.	"	6:4	"	"	"	"
9.	"	5:5	"	oil		"

In fraction 1 to 5 crystals of methyl cholanate were eluted which were sublimed and gave no depression of a mixture melting point with crystals(chromatogram 16, fraction 1 to 4) assumed to be methyl cholanate.

45. Hydrogenation of 9:11-lithocholenic acid.(LVIII).

Lithocholenic acid(25.2 mg) was shaken in the micro hydrogenation apparatus in acetic acid(1 ml.) with hydrogen and platinum oxide(6 mg.) as catalyst. The volume of hydrogen used up, after allowance had been made for leakage, amounted only to 11% of theory for the saturation of one double bond.

The solution was filtered, the filtrate was diluted with ten volumes of water and extracted with ether. On distillation of the washed and dried ether, crystals were isolated, which melted at 186-187°C. As lithocholic and 9:11-lithocholenic acid have the same melting point and do not show a mixture melting point depression, the crystals were methylated with diazomethane. Methyl Δ9:11-lithocholenate, m.p. 112-114°C, (LIX), was isolated and identified.

46. Hydrogenation of 9:11-lithocholenic acid methyl ester, (LIX).

The ester(LIX)(25.4 mg.) was shaken in the presence of hydrogen and platinum oxide as catalyst, as described above. Only ten percent of the theoretical uptake of hydrogen was used, after corrections had been made for leakage, temperature and pressure. The solution was filtered, diluted and extracted, and crystals were obtained showing the characteristic melting point of methyl 9:11-lithocholenate(LIX), m.p. 110-112°C. (The melting point of methyl lithocholate is given as 130°C.)

Part 1 C .

The bromination of bisnordesoxycholic acid and etio-allocholanic acid.

1. Introduction.

The bromination of fatty acids in the α-position has frequently been described in the literature. Gilman(467) quotes the preparation of the α-bromo derivative of caproic acid,using bromine in the presence of phosphorus trichloride. Schmidt(468) halogenated acids,with red phosphorus serving as catalyst. According to Richter(469) halogenation of the acid chlorides proceeds more rapidly than that of the corresponding acids, postulating the acid halide formation as an intermediate in the halogenation reaction. The preparation of α-bromo-palmitic acid has been described by Hickinbottom (470),who obtained the compound by reacting dry bromine with palmitic acid in the presence of red phosphorus. Houben(471), Zelinski(472) and Vollhart(473) report brominations of fatty acids using phosphorus pentabromide or red phosphorus as catalyst.

Enolisation of the acid halide, followed by addition of bromine and elimination of a molecule of hydrogen bromide, has been proposed as the mechanism of the reaction. The presence of a phospho-halide appears to be necessary.

The bromination of a bile acid in the 4-position has never been accomplished. Wallis(474) described the action of bromine on the silver salts of bile acids, which resulted in the decarboxylation of the compound and the introduction of a bromine atom on the terminal carbon atom with precipitation of silverbromide. This type of reaction is, however, different from the one under consideration in this investigation.

Fleischer(475) prepared the aldehyde corresponding to desoxycholic acid and Gallagher(476) synthesized the nor-cholanyl methyl ketone from desoxycholic acid. Both compounds could be brominated in the 23-position in the presence of hydrogen bromide. On debromination with collidine and oxidation, bisnordesoxycholic acid was obtained, although in poor yield.

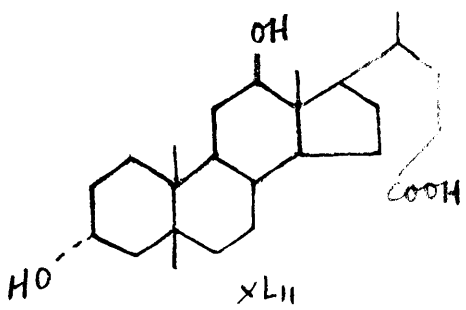
These reactions, involving the bromination of a methylene group next to a carbonyl double bond, have been applied with great success to other positions in the steroid molecule. The reaction was discovered by Butenandt(404), who introduced a bromine atom in the 4-position adjacent to a 3-ketone group. Marker and Wintersteiner(478,479) described the bromination in C_{11}-position, and the bromination of the C_{17} and C_{21}-positions adjacent to ketonic groups was also investigated by Marker(477). The bromine in most of these compounds could be displaced by a hydroxyl group on saponification, an important factor for this study.

2. Discussion.

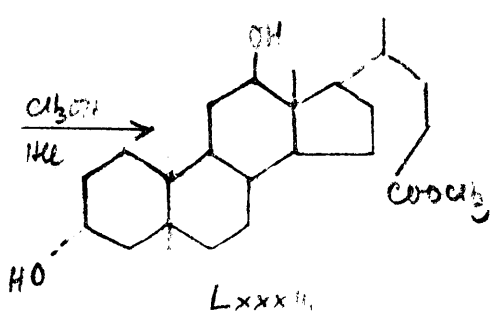
The bromination of bisnordesoxycholic acid was investigated in an effort to shorten the degradation of the bile acid sidechain. In the Barbier-Wieland(457,458) step-wise degradation(LXXXIII-XCIV), the nor-(LXXXVII) and the bisnor acid (LXXXVIII) were obtained in good yields. Kendall(459) achieved 90% yields of the two acids by a two phase oxidation in acetic acid, chloroform and sulphuric acid at room temperature.

However, the further degradation of the bisnor acid to the etio acid (XCIV) by the method of Hoehn and Mason(460) proved tedious and unsuited for large scale production,on account of the difficulties in handling large amounts of ozone and periodic acid.Only 33% yields were obtained by Heard and Wasson(461) by running the six steps from the ternorcholanyldiphenylethylene (LXXXIX) to the etio acid(XCIV) without isolation of the intermediates. The literature on the modifications and improvements of the sidechain degradation is considerable(460-466), the yield,however, still poor. Direct oxidation ofdesoxycholic acid diacetate and of desoxycholic acid with the bile acid sidechain replaced by the sterol type sidechain under more vigorous conditions(Ruzicka(453,454) was attempted without success (455,456,462).

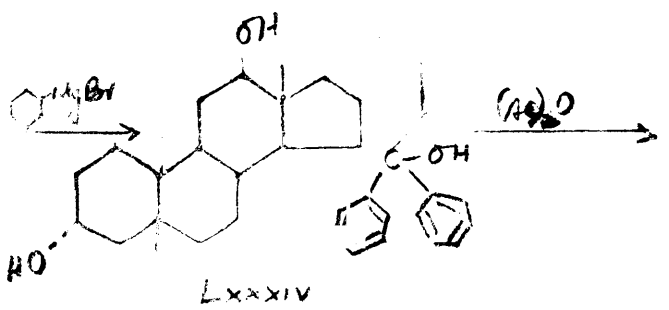
Barbier-Wieland Degradation.



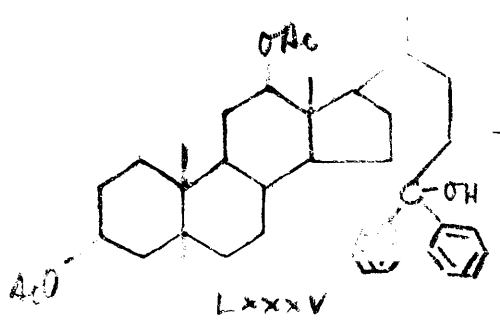
XLII



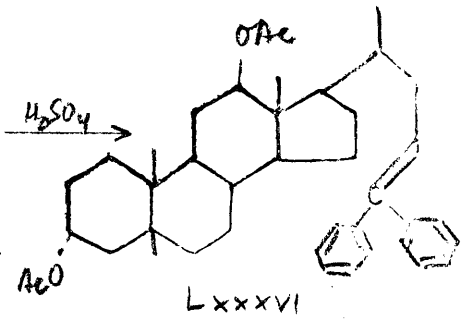
LXXXIII



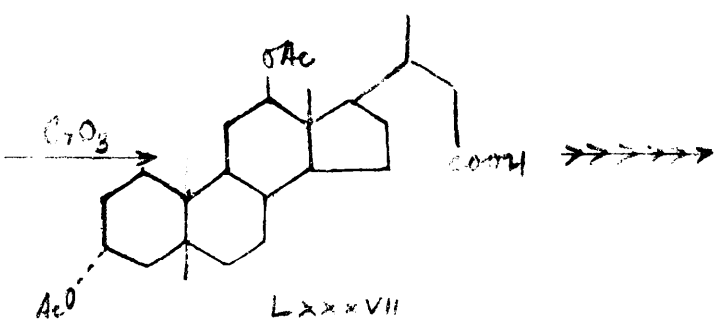
LXXXIV



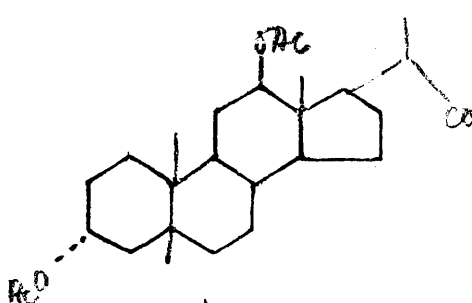
LXXXV



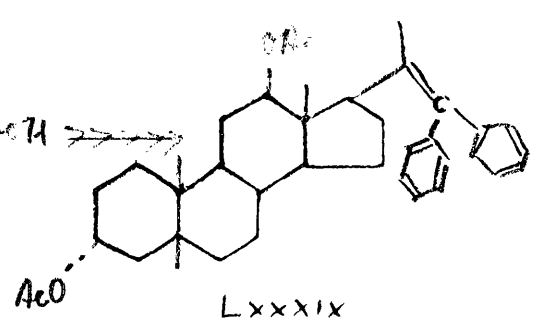
LXXXVI



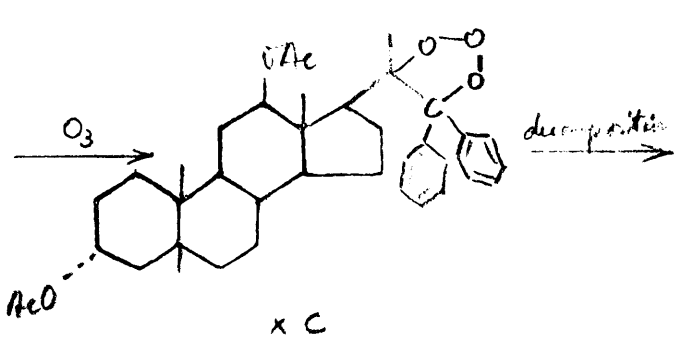
LXXXVII



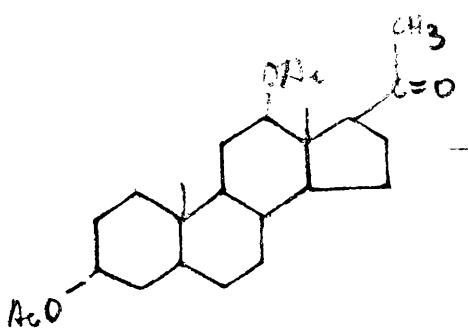
LXXXVIII



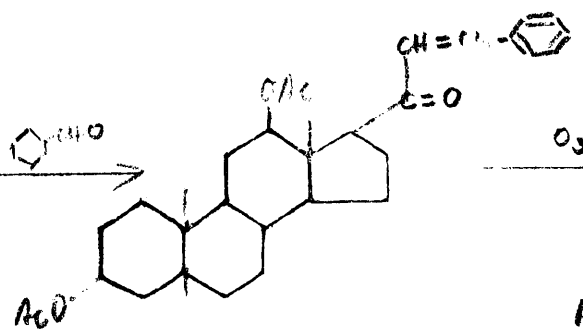
LXXXIX



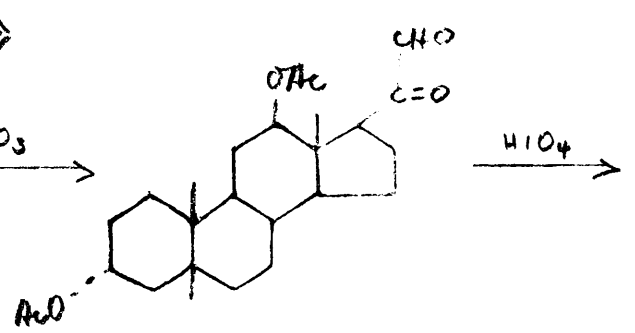
XC



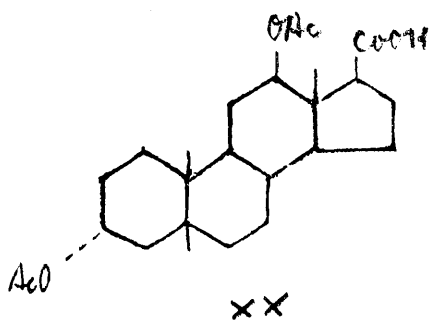
XCI



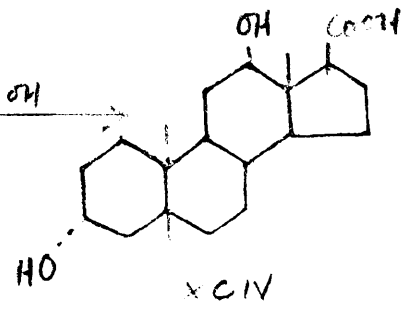
XCII



XCIII



XX

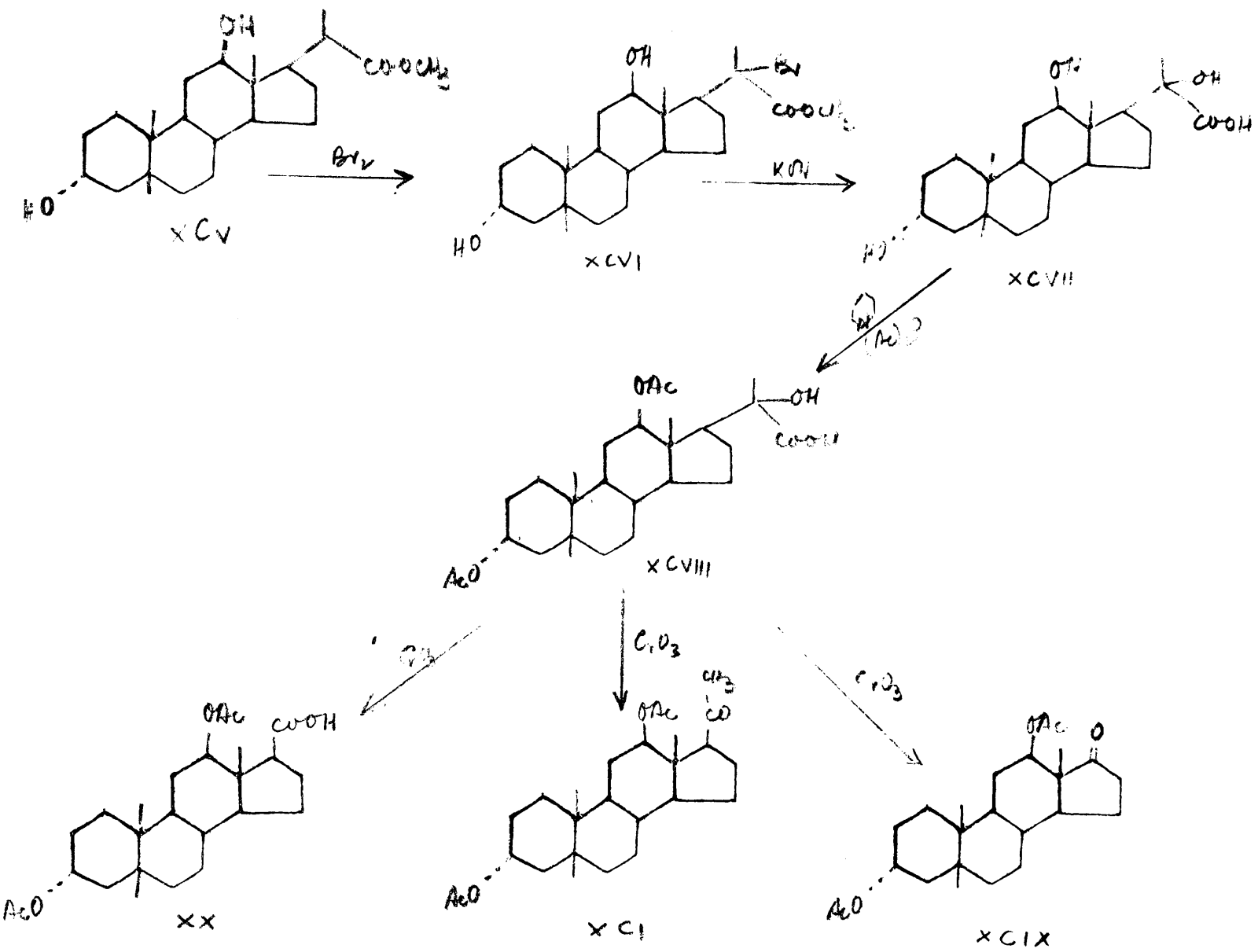


XCIV

The introduction of an atom of bromine in the 20-position, followed by saponification, acetylation and chromic acid oxidation would present a rapid and simple method of obtaining the etio acid together with the C_{17} and C_{20}-ketones. (XCV - XCIX).

The bromination of bisnordesoxycholic acid (C) in acetic acid was attempted, but decoloration of the bromine solution could not be observed. When the bromine solution was added to a carbon tetrachloride solution of methyl bisnordesoxycholate (XCV), refluxing on the steam bath, decoloration of the bromine occurred and the crystalline methyl bisnordesoxycholate was not recovered. On saponification crystals were obtained, which were identified as unchanged starting material. From the mother liquors crystals were isolated which showed a different melting point: 148-150°C, and, at that time, were assumed to be a new compound. However, on drying in the desiccator for several weeks, these crystals also showed the melting point of bisnordesoxycholic acid (212-214°C) and did not give a depression of a mixture melting point with compound (C).

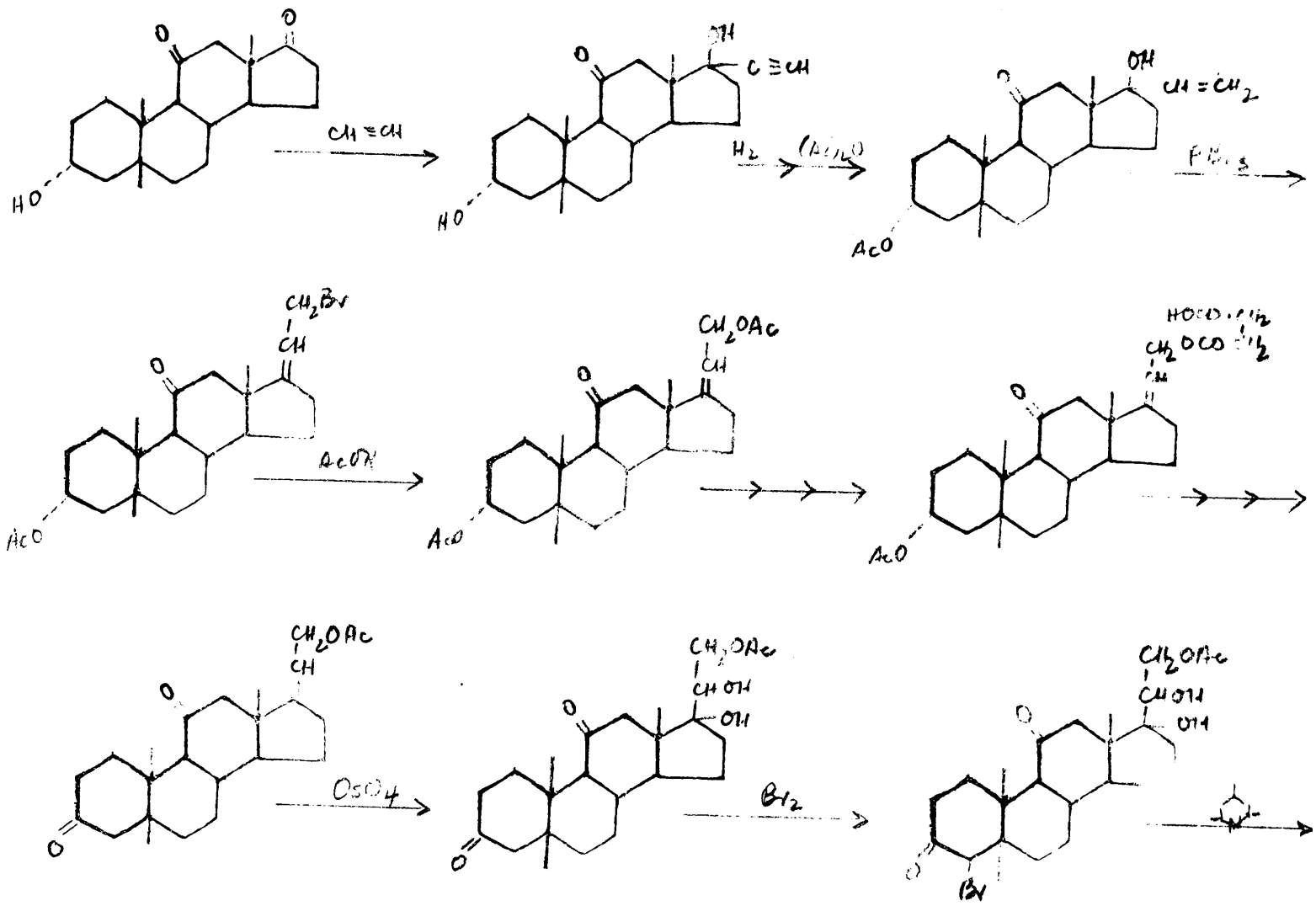
Before the identity of the low melting variety of bisnordesoxycholic acid was established, a number of attempts were made, to increase the yield of that compound. As large amounts of unchanged starting material were recovered after saponification, bromine was added, until decoloration no longer occurred, (2 moles) without improving the yield. Identical results were obtained when red phosphorus

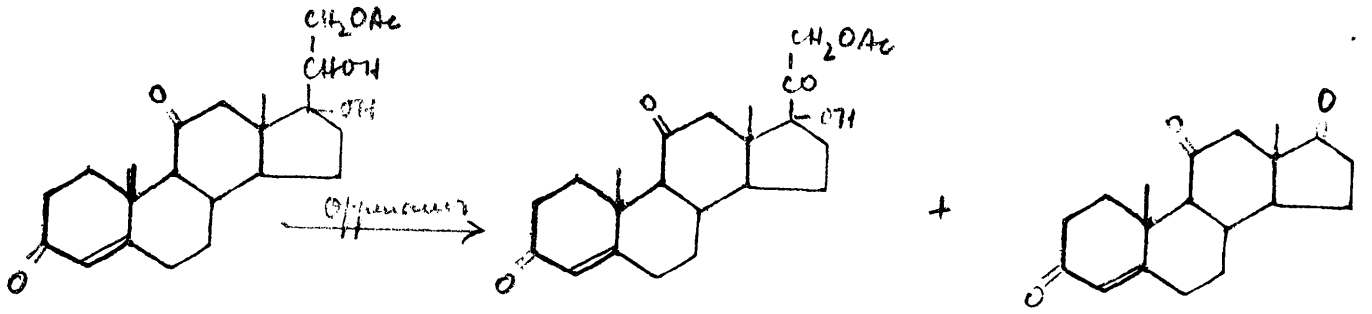

OH
HO
XCV
XCVI
XCVII
OAc
AcO
XCVIII
XX
XCI
XCIX
C
CI
CII
CIII
XCVII

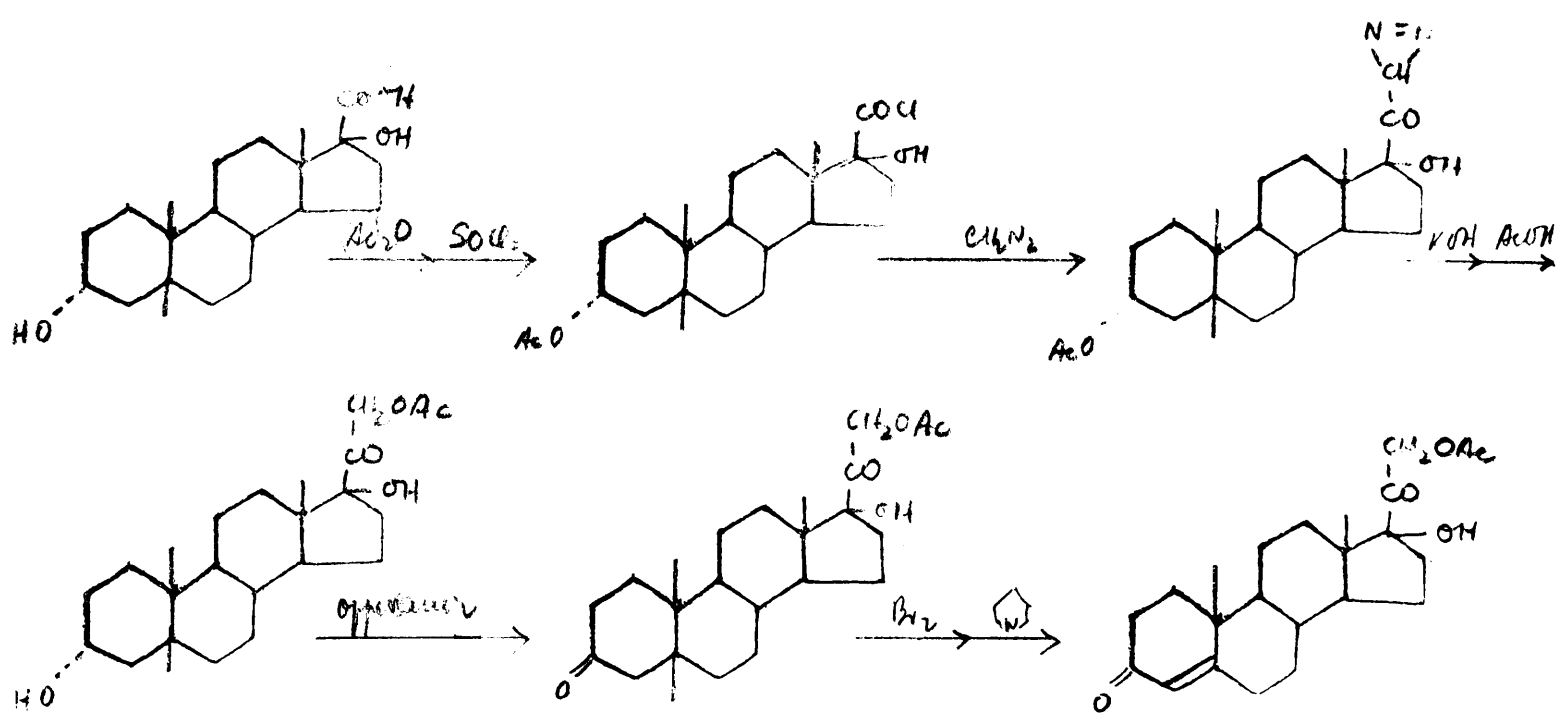
was used as catalyst. Following Richter's suggestion, the bromination of the acid chloride was investigated. When thionyl chloride was added to 3,12-diacetoxy-bisnorcholanic acid, followed by bromination and saponification, only unchanged starting material, bisnordesoxycholic acid, could be recovered. Decoloration of the bromine solution had also not occurred. Under these conditions the anhydride, instead of the acid chloride, is formed, explaining the lack of reactivity. When the bisnor acid was added to the thionyl chloride solution, the acid chloride was formed, and the bromine solution was decolorized on addition. Saponification yielded again bisnordesoxycholic acid in both allotropic forms. The introduction of a bromine atom in the α-position of the bisnor acid sidechain had not been accomplished.

Bromination of a bile acid was again investigated, in an attempt to prepare adrenal cortical hormones of the type of Kendall's compound "E". The synthesis of the dihydroxy acetone sidechain had been accomplished by Reichstein, Kendall and Sarett (411). As starting materials for the sidechain synthesis, etiocholane-3-ol-17-one and acetylene were used.

Starting from an etio acid, it was hoped, bromination and saponification would yield the required 17-hydroxy-etio acid, which could then be transformed in good yield to the compound with a dihydroxy-acetone sidechain by the chain of reactions used in part 1 **A** of this thesis.

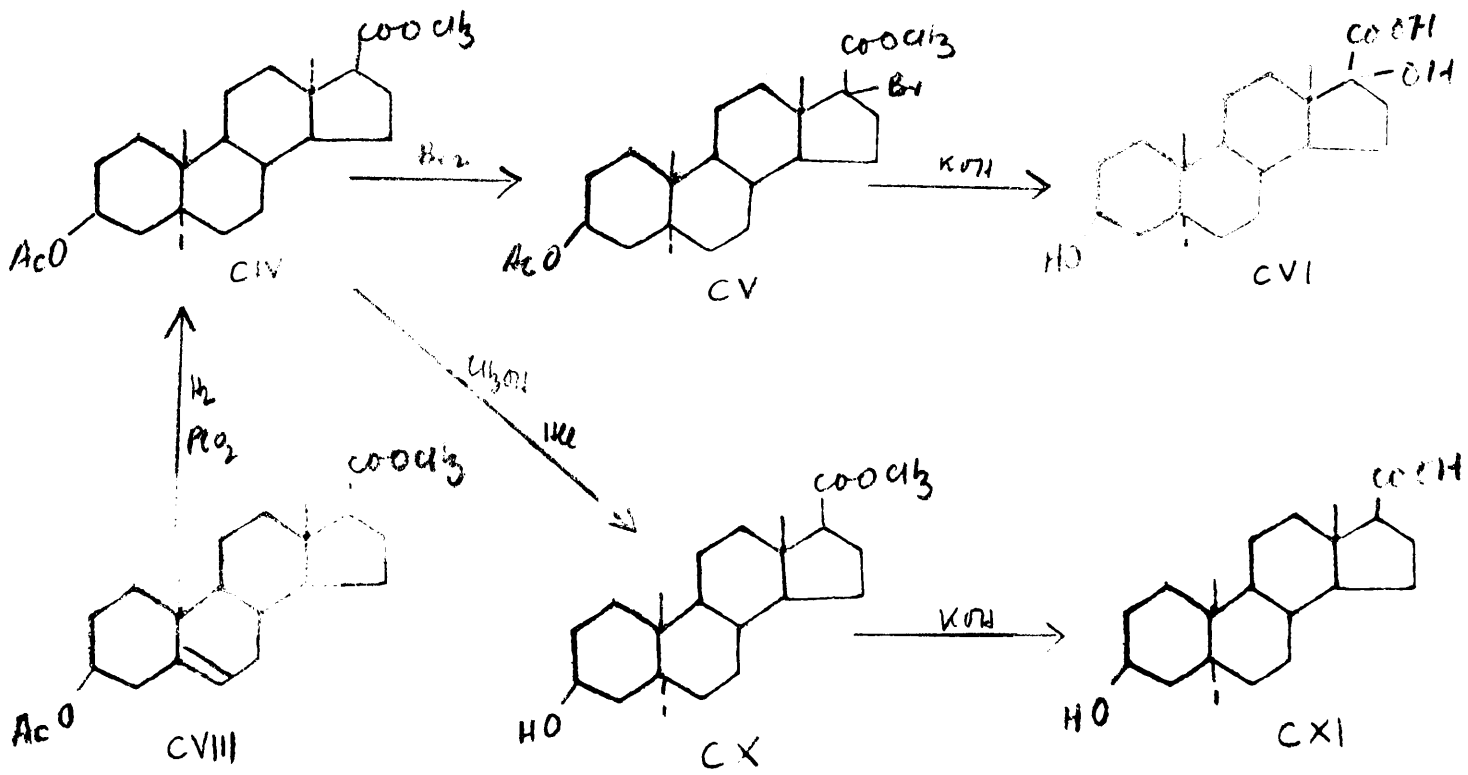






3-(Β)-ol-Δ5:6-etiocholenic acid was reduced with hydrogen and platinum oxide as catalyst to 3-(Β)-ol-etioallocholanic acid and to a small degree to the C_5-epimer. Bromination of methyl 3-(Β)-acetoxy-etioallocholanate(CIV) was carried out in acetic acid in the presence of dry hydrogen bromide. A mixture of crystalline compounds was obtained.

Partial hydrolysis of the starting material was suspected and 3-(Β)-ol-etioallocholanic acid methyl ester (CX) was prepared by hydrolysis in methanolic hyarochloric acid. Another sample of starting material was saponified to 3-(Β)-ol-etioallocholanic acid (CXI). The compounds isolated after bromination were found to be identical with these two hydrolytic products. On saponification of the bromination products, 3-(Β)-ol-etioallocholanic acid was obtained. Bromination had not occurred. The investigation was therefore discontinued.



3. Experimental Work.

1. Action of bromine in acetic acid on bisnordesoxycholic acid(C) at room temperature.

Bromine (4.4 g.) was dissolved in acetic acid, and the solution was diluted to 50 ml. in a volumetric flask. The strength of the bromine solution was determined by titration, described in part 1 A, section 16. Five ml. of the bromine solution per g. bisnordesoxycholic acid were one molar equivalent.

Bisnordesoxycholic acid(C)(1.03 g.) was dissolved in glacial acetic acid(10 ml.), five drops of the bromine solution were added, and the solution was allowed to stand at room temperature. Decoloration was not observed. After the addition of one mole bromine, the solution was allowed to stand for 16 hours at room temperature. The mixture was diluted with ten volumes water, the bromine removed, and unchanged starting material was recovered from the ether extract.

2. Action of bromine in carbon tetrachloride on methyl bisnordesoxycholate (XCV) on the steam bath.

A solution of 4.4 g. bromine in 50 ml. carbon tetrachloride was prepared and standardized as described. The ester (XCV)(958.5 mg.) was dissolved in carbon tetrachloride(25 ml.), and 5 ml. bromine solution(1.1 mole) was added to the gently boiling solution, dropwise, awaiting decoloration before

further addition of bromine. In the absence of the bisnordesoxycholic acid methyl ester, decoloration of the bromine solution on addition to boiling carbon tetrachloride was not observed.

The brominated solution was cooled, washed with 10% sodium carbonate solution(3x20 ml.), with water until neutral, dried with sodium sulphate and taken to dryness. The brown residue could not be crystallized.

Bromination was repeated on a smaller scale on the ester(213 mg.) until decoloration of the bromine did not occur anymore.(2 moles bromine).On extraction a brown oil was obtained. The same results were obtained from a bromination of the ester in the presence of red phosphorus as catalyst.Bromine was decolorized and fumes of hydrogen bromide were given off(trapped in water) in all experiments.Crystallisation of the oily residue never succeeded.

3. Saponification of the bromination product.

The oils obtained on bromination of the bisnordesoxycholic acid methyl ester were separately saponified by reflux for one hour in methanolic potassium hydroxide (5 g. in 20 ml.90% methanol). The cooled solutions were poured into ten volumes of ice water, filtered, acidified with dilute hydrochloric acid, and the precipitate was filtered,

washed, dried and dissolved in methanol. On addition of ether to the methanolic solution, crystals formed, m.p. 208-212°C, which were filtered. From the filtrate on addition of ether, more crystals of the same compound separated. They were filtered off, and the filtrate was condensed to a small volume, diluted with benzene and allowed to stand in the refrigerator. Crystals were filtered off which melted at 188-193°C. Two further crops of crystals were isolated in the same way from the filtrates. m.p. 148-149°C.

To prevent secondary reactions of the bromination product on exposure to light, a further batch of the crystals (XCV) (1.012 g.) was brominated, and the reaction product was saponified. On fractional crystallisation the two types of crystals were again isolated.

The first two crops of the saponification products crystallized readily, m.p. 208-212°C and were identified as bisnordesoxycholic acid. The next crop showed a broad melting point, while the last two or three crops melted sharply at 148-150°C. This compound was not the methyl ester of bisnordesoxycholic acid, as was suspected (methylation of the acid in methanol with traces of hydrogen chloride), but was identified as bisnordesoxycholic acid, after desiccation for several weeks, m.p. 208°C. It is assumed to be an allomorph of bisnordesoxycholic acid.

4. Acetylation of bisnordesoxycholic acid.(C).

The acid (C)(0.9703 g.) was dissolved in acetic anhydride(13 ml.) and pyridine(16 ml.) and allowed to stand for 48 hours at room temperature under anhydrous conditions. The solution was then heated for 30 minutes on the water bath, water (3 ml.) was added, and the heating on the steam bath was continued for two hours, to decompose the anhydride formed. The solvent was removed on the steambath under vacuum, the residue was taken up in ether(50 ml.), washed with dilute hydrochloric acid and extracted with ice cold 5% sodium carbonate solution(3x30 ml.)The extracts were quickly acidified and extracted with ether. The ethereal solution was washed, dried and taken to dryness. The residue crystallized from ether, m.p.155-160°C.

5. Bromination of bisnordesoxycholic acid diacetate after treatment with thionyl chloride.

Thionyl chloride (5 ml.)(purified by distillation from quinoline and boiled linseed oil) was added to the diacetate (CI)(1 g.) at 0°C, and the solution was allowed to stand for one hour at 0°C and 22 hours at room temperature under anhydrous conditions. The solvent was then distilled under vacuum below 40°C. The residue was dissolved in glacial acetic acid(10 ml.) and 5 ml. of the solution of bromine in acetic acid was added slowly. Decoloration of the bromine solution did not occur.

The solution was extracted and the resiaue was saponified. Pure crystals of bisnordesoxycholic acid were recovered, m.p.212-214°C.

6. Bromination of 3,12-diacetoxy-bisnorcholanyl chloride.(CI).

The diacetate(CI)(962.1 mg.) was added to the thionyl chloride (5 ml.) at 0°C, and the solution was kept for 16 hours in an ice bath under anhydrous conditions. The solution was diluted with dry carbon tetrachloride (5 ml.), and 5 ml. of the bromine-in-carbon-tetrachloride solution was added dropwise. After three hours, all the bromine had reacted, and the solvents were removed under vacuum at 50°C. The residue was saponified with 20% methanolic potassium hydroxide solution(one hour refluxing). On extraction, an oil(884.3 mg) was obtained which crystallized from ether, petrol ether. m.p.143-150°C.

7. Methylation and acetylation of product of section 6.

The crystals(800 mg.) were dissolved in ether and methylated with diazomethane. On evaporation of the ether and destruction of excess diazomethane, an oily residue was obtained. It was dried, dissolved in acetic anhydride (10 ml.) and anhydrous pyridine(12 ml.) and allowed to stand at room temperature for 60 hours under anhydrous conditions. The solution was poured into ten volumes acidified ice water and extracted with ether(3x100 ml.)From the ether extract an oil was obtained. On chromatography only oils were eluted.

Bromination of 3-(β)-ol-etioallocholanic acid.

8. Acetylation of 3-(β)-ol-Δ4:5-etiocholenic acid methyl ester.

The ester (5 g.)(m.p.180-183°C) was dissolved in acetic anhydride(27 ml.) and heated for 30 minutes on the steam bath under anhydrous conditions. On cooling crystals separated and were filtered. The filtrate was diluted with ten volumes of water, and the crystals formed were also filtered. The 3-acetate (CVIII), m.p.155-157°C was obtained in quantitative yield.

9. Hyarogenation of 3-(β)-acetoxy-Δ4:5-etiocholenic acid methyl ester.(CVIII).

The crystals (CVIII)(4.9 g.) were dissolved in acetic acid (50 ml.) and platinum oxide(150 mg.) was added. The compound was reduced as described for the micro hyaro-genations in part 1 B, section 28. After one hour 330 ml. hydrogen gas had been used up and further uptake was not observed. 110% of the calculated amount of hydrogen for the saturation of one double bond had been used. The solution was filtered(to recover the platinum), and the filtrate was diluted with ten volumes ice water. Crystals precipitated which were filtered, washed and recrystallized from methanol. The crystals of the first two crops melted at 155-154°C. (CIV) From the mother liquors crystals of the C_5-epimer separated.

10. Partial hydrolysis of 3-(B)-acetoxy-etioallocholanic acid methyl ester(CIV).

The compound(CIV)(97 mg.) was dissolved in methanol (10 ml.) containing concentrated hydrochloric acid (0.1 ml.). After standing at room temperature for 24 hours, the solution was diluted with ten volumes of water and extracted with ether(3x30 ml.) The ether solution was washed neutral with water, dried with sodium sulphate and taken to dryness. The residue crystallized from methanol, m.p.175-177°C.(CX).

11. Saponification of 3-(B)-acetoxy-etioallocholanic acid methyl ester(CIV).

Saponification was carried out on 93 mg. of the ester(CIV) in methanol(10 ml.) and 6 N sodium hydroxide solution(5 ml.)(2 hours refluxing). The solution was cooled, diluted with ten volumes water, washed with ether, acidified and extracted with ether(3x50 ml.). The washed and dried ether extract yielded crystals on evaporation of the solvent. m.p.248-250°C.(CXI).

12. Bromination of 3-(B)-acetoxy-etioallocholanic acid methyl ester.(CIV).

Dry hydrogen bromide was liberated by the addition of bromine to tetralin and dissolved in glacial acetic acid, (directions found in Houben).

The ester(CIV)(997.7 mg.) was dissolved in 10 ml. acetic acid, saturated with dry hyarogen bromide, and a few drops of the bromine-in-acetic-acid solution were added. At room temperature no decoloration could be observed, even after irradiation with ultraviolet light. On heating the solution on the steam bath decoloration was observed and the rest of the bromine solution was added dropwise.(1 mole). After 4 hours the solution was cooled, diluted with ten volumes of water and extracted with ether(3x50 ml.).Extraction with 5% sodium carbonate solution had to be abandoned on account of the formation of unbreakable emulsions. The emulsion was broken on acidification, and the material was reëxtracted with ether.The ether was washed, dried and distilled.Crystals formed.They melted partly at 178-180°C and the rest melted at 205-212°C. Hydrolysis of the 3-acetoxy group during bromination and sodium carbonate extraction was suspected to have occurred.The crystals were, therefore, saponified.

13. Saponification of bromination product.

The crystals were dissolved in methanol(26 ml.) and 6 N sodium hydroxide solution (13 ml.) and refluxed on the steam bath for two hours. The red solution was diluted with ten volumes of water, washed with ether and acidified. The yellow precipitate was filtered, washed with water and crystallized from methanol. The crystals, m.p. 246-249°C,

showed no depression of the melting point when mixed with 3-(β)-ol-etioallocholanic acid(CXI).

14. Methylation of crystals obtained in section 13.

The crystals, melting at 246-249°C, (150 mg.) were suspended in ether and methylated with diazomethane. After 15 minutes at room temperature, the diazomethane and ether were distilled. Crystals, melting at 175-178°C, were isolated. They showed no melting point depression when mixed with 3-(β)-etioallocholanic acid methyl ester and with the crystals isolated after bromination, m.p.178-180°C.

Summary and Conclusion of Part 1.

A. The difference in the yield of pregnane-21-acetoxy-3,12,20-trione, whether prepared from 3,12-diketo- or 3,12-diacetoxy-etiocholanic acid, was found to be due to the interference of ketonic groups in the formation of the acid chloride with thionyl chloride.

12-keto-desoxycorticosterone acetate was prepared by the method of Fuchs and Reichstein and found to be inactive in the glycogen deposition and the Ingle test. In larger doses (0.5 mg.) the compound was toxic.

B. The preparation of 3-hydroxy-12-keto-cholanic acid was studied by partial acetylation, succinoylation and semi-phthallate formation as intermediate reactions. With selenium dioxide, methyl 3-acetoxy-12-keto-cholanate was dehydrogenated, and the separation of the 50%pure reaction product from its starting material was studied. Prolongued dehydrogenation with additional amounts of selenium dioxide and periodic spectrophotometric examination of the product gave 95% yield of the desired compound. The product did not react with semi-carbazide under any condition, but the semi-carbazone was formed after hydrolysis to the free acid. On Wolff-Kishner reduction with sodium benzylate, Δ9:11-lithocholenic acid was isolated.

Nitration of Δ9:11-lithocholenic acid does not produce 11-nitro-Δ 9:11-lithocholenic acid.

The C_3-nitric acid ester is formed, which on hydrolysis gives rise to the C_3-epimer of 9:11-lithocholenic acid ester. Epimerisation was proven by preparation of a number of derivatives, which could be identified, by repetition of the reactions with lithocholic acid and by digitonin precipitation of the ß-hydroxyl epimers at C_3.

Dehydration produces an unsaturated linkage in the 2:3 or 3:4 position on hydrolysis of the nitrate, and the small amount of this product was identified after reduction to methyl cholanate by mixed melting point determination with methyl cholanate, produced from desoxycholic acid.

The observation was made, that the reduction with platinum oxide as catalyst proceeds readily in case of the C_3-epimers of Δ 9:11-lithocholenates, but not in the case of 9:11-lithocholenate or the corresponding acid. The importance of the position of the C_3-hydroxyl to the reactivity of the 9:11 double bond during nitration requires investigation.

C. Bromination of bisnordesoxycholic acid, its acid chloride and methyl ester and of C_3-epi-etioallocholanic acid at the α-carbon atom was studied under various conditions. The desired bromo compound, respectively their saponification product, the α-hydroxy acid, could not be obtained.

Part 2.

The Application of Ultraviolet Spectrophotometry to the Estimation of Steroid Hormones in Blood and Urine.

1. Introduction.

At present, the accurate estimation of progesterone and estrogens in tissues and fluids depends on biological assay. The original method of Corner and Allen(354,355) for the estimation of progesterone has been modified and improved (356,357), so that now quantitative estimation of micrograms of progestational hormone is possible.(358-361). For the determination of estrogen the vaginal smear technique of Allen and Doisy (281,282) has proved very valuable. Since then a number of more rapid biological assays have been developed, and the methods have been standardized to give uniform results.(278-286).

In all bioassay methods a number of variable factors in the standardisation of hormones exist, which have been studied in the case of the estrogens, by Marrian and Pugsley. (288-290): The variation in the reaction of individual mice to the injection of the same amount of hormone is considerable, even amongst fairly homozygous animals. Similar, though less, variation is found in the reaction of groups of 20 animals. The condition of the animal (age, weight and health) is also of importance. Regarding the method of administration, division of the dose into a series of injections, and lengthening of the

period of administration to 24 to 36 hours nearly doubles the percentage response. The solvent and the site of injection are also of significance. The criteria of estrus vary from the disappearance of leucocytes to the complete cornification of the vaginal contents. Another important factor is the number of smears taken, because short periods of cornification can be missed on daily examination, while,on the other hand,positive reactions can be elicited on frequent smearing in the absence of estrogens.(279). Emmens (291) showed, that non-estrogenic substances,existing in urine,can increase estrogen activity ten-fold, while Marker (292) discovered the presence of allopregnanediol in urine, which possesses androgenic properties, counteracting the effect of the estrogens. When larger quantities of hormone are present, non-phenolic compounds can be eliminated, and the estrogens are partitioned into strong and weak phenolic estrogens. Small errors in the distribution of the hormones are, however, multiplied on bioassay, due to the difference in biological activity of the estrogenic hormones, and have invalidated scores of experimental data.

The need for a simple chemical method started the investigation of colour reactions for the estimation of hormones.(293-303). Most of them have proved to be non-specific, but the Zimmermann reaction (303) for the estimation of 17- and 20-ketones and the Kober test (304-315) for estrogens have been found sufficiently sensitive and specific to be useful.

The amounts of estrogenic and progestational hormones, present in blood, are too small to be detected colorimetrically. Thorough investigations of the quantities of estrogens in blood during the menstrual cycle and pregnancy, have revealed the presence of ½ mg. estrogen (calculated as estrone) in 100 ml. of blood. According to Szego (343,344,145), the estrogens are in a water soluble form, 2/3 of which are in loose combination with protein. The work of Hoffmann(365-368) has established the existence of 50 to 100 microgram of progestational hormone in 100 ml. of serum during advanced pregnancy. Its physical and chemical nature has not yet been determined. Polarographic determination of progesterone(Heyrovsky, Wolfe et al., 369-371) is thought to be not sufficiently sensitive for such minute amounts.

The liberation of the estrogens from their conjugation in blood (333-342) and urine (218) is a prerequisite for their chemical estimation. A variety of methods for the hydrolysis of estrogen conjugates have since been published. (219-256). Under all conditions two processes, the splitting of the conjugate and the destruction of the liberated estrogen, proceed simultaneously (231,232,253,254). The method, giving maximum hydrolysis and minimum destruction, was developed by Larrian (230).

When the Kober reaction is applied to such urine extracts, the formation of a pigment giving a brown colour with the reagent, interferes with the accurate determination

of the estrogens present. Many investigations have been made to eliminate or correct for these interfering substances — not obtained on hydrolysis above pH 4 (Schmulowitz, 237) — without striking success. (304-315). Only after the 4th month of gestation, the Kober test becomes reliable.

Because of these limitations of existing methods, the spectrophotometric determination of small quantities of progesterone and estrogens was explored. The high molar extinction coefficient of α,β-unsaturated ketones at λ_{max} 240 mμ is sufficient to detect 3.8 μg progesterone in 2 ml. of solvent with accuracy. The determination of progesterone and other α,β-unsaturated ketones in 5 ml. of blood should, therefore, be practicable.

With the estrogenic hormones, the weaker molar extinction coefficient (2000) at λ_{max} 280 mμ demands a higher concentration of the hormones. 40 ml. of blood would be required, provided destruction and pigment formation on hydrolysis could be eliminated.

For the determination of estrogens in urine during the menstrual cycle and pregnancy, this method appeared to present great possibilities, as adequate quantities of urine are easily obtained. The investigation, originated by Reynolds (373), on the microdetermination of progesterone and estrogens by ultraviolet spectrophotometry was, therefore, applied to biological fluids.

2. Discussion.

The extraction of lipids from serum had to be studied first. Reynolds (373) followed Allen's method (372) for the extraction of progesterone from ovaries. Although Allen succeeded in isolating progesterone, the addition of alcohol to serum was not considered sufficient for the complete removal of lipids from their possible combinations.

Macheboeuf and Tayeau (379-383) described the liberation of lipius from blood by means of soaps, particularly potassium bromo-stearate. Chargaff (378) succeeded in liberating most lipids from protein combination by the use of heparin, but complete release was not effected. The two most promising methods described in the literature are a) the quick-freezing of serum emulsified with ether (Chargaff and McFarlane, 384-386) and b) the methanol extraction of lyophilized serum (Flosdorf et al.)(377).Both methods were examined in this investigation.

The ether extracts (method(a)) were washed with N sodium hydroxide solution to remove acids and phenols, washed neutral with water and evaporated to dryness. The methanol extracts (method(b)) were taken to dryness, and the residue was dissolved in ether and purified as in method(a). The dried residue was treated with Girard's reagent "T" and the ketonic fraction was examined spectrophotometrically for the presence of a 240mμ absorption maximum.

In a preliminary investigation, the absorption spectrum and the molar extinction coefficient at λ_{max} 240 mμ (16600) of pure progesterone in ethanol were recorded.

To study the recovery of progesterone from serum, four mg. progesterone were added to dried serum and extracted as described above. 90% of the added material was recovered. A similar recovery experiment, to test further the accuracy of the method, was carried out on dried serum, to which 50 mg. cholestenone had been added. 90% of the material was recovered in the ketonic fraction. A second separation of ketonic material and a longer period of hydrolysis increased the total yield by 2.7%.

The preliminary findings justified the application of the method to the determination of progesterone in serum. A sample of pooled lyophilized plasma was extracted and analyzed spectrophotometrically. A maximum at 240 mμ was not observed; instead a strong absorption band at 230 mμ suggested the presence of other unsaturated ketones, interfering with the estimation of progesterone.

To examine the interfering effect of the λ_{max} 230 mμ absorbing compound, progesterone was added in increasing doses to the plasma extract. As the amount of progesterone was increased, the absorption maximum was observed to shift from 230 to 240 mμ and their extinction coefficients were found to add up.

The elimination of the interfering substance by chromatographic separation was investigated. Progesterone, which had been recovered from one of the preliminary experiments, was chromatographed on a column of alumina and was quantitatively recovered on elution with benzene 100% and benzene to ether 20:1 mixtures.

An extract of lyophilized plasma was chromatographed, and all eluates were examined spectrophotometrically. In the majority of fractions the 280 mμ maximum was observed. At 240 mμ no absorption band could be observed in any of the fractions. With 100% benzene a small amount of crystalline material was eluted, which showed an absorption band at 275 mμ and melted at 144°C. This substance was isolated from every ketonic plasma extract in small amounts, but was not identified. (Pregnanolone ?)

As progesterone could not be discovered in normal plasma, one ml. samples of plasma of a woman in advanced pregnancy were examined. A 240 mμ maximum was not detected.

Blood plasma, blood serum and laked blood cells gave all the same results: a strong absorption band at 280 mμ was found in all extracts, but a 240 mμ maximum could not be discovered even after chromatographic separation.

In case of a stronger, chemical bond between the hormone and plasma proteins, plasma was acidified with concentrated hydrochloric acid and extracted and fractionated.

Crystals melting at 144°C were again obtained, together with oily fractions absorbing maximally at 230 and 234 mμ. however, in addition an absorption maximum at 238 mμ and two at 240 mμ were discovered. As a 100 ml. plasma was used for this investigation, less importance was attached to the strong acid treatment than to the use of large enough quantities of serum or plasma. From the extinction coefficient, the concentrations of α,β-unsaturated ketones was calculated. From 100 ml. pooled plasma 67 μg α:β-unsaturated ketones were isolated. Their identification as testosterone, progesterone or adrenal cortical hormones was not undertaken. However, model chromatograms of the individual crystalline hormones and their mixtures can be expected to give an elution pattern, from which the fractions of serum extracts could be identified.

----oooooooooo-----

The estrogen estimate (bioassay) of normal non-pregnancy urine varies from 30 to 300 μg estrogen (calculated as estrone). As all estrogenic material is presumably excreted in a water soluble form, hydrolysis of the conjugates appears to be necessary. The method of hydrolysis adopted in the present investigation is that of Smith and Smith(247): refluxing for ten minutes with 150 ml. concentrated hydrochloric acid per liter of urine.

The estrogens were extracted with ether, taken to dryness and partitioned by the method of Bachman(185) and

Mather(186). This method proved to be superior to that of Marrian (182). The "weak phenolic" fraction was divided into non-ketonic and ketonic portions by Girards reagent "T". The three fractions were analyzed spectrophotometrically, but an absorption maximum at 280 mμ could not be detected.

Chromatographic separation of the three estrogen fractions was used to eliminate interfering substances. Model chromatograms on samples of all three estrogens indicated, that estrone and estradiol were eluted with 15% acetone in petrol ether, while estriol was obtained on elution with 100% ether. The weak, non-ketonic phenols of the urine extract did not show a maximum in the region of 280 mμ in any of the eluates. The ketonic, weak phenols showed one maximum at 286mμ, and the strong phenols showed one band at 280 mμ. As considerable amounts of interfering substances were still present, purification by sublimation was attempted.

The "estradiol" fraction did not yield a sublimate absorbing maximally at 280 mμ, confirming the absence of estradiol from this fraction. The "estriol" fraction gave rise to a sublimate, which showed an absorption band at 280 mμ (probably due to p-cresol) and did no longer show a maximum at 280 mμ in the unsublimed residue.

Because of the large amount of interfering substances, the accuracy of the determination of small quantities of estrogens in the presence of these impurities was investigated. On

addition of 15 to 120 µg. of estriol to the impurities (eluted with the solvent mixture, expected to remove estrone from adsorption on alumina), the absorption maximum was observed to shift from 280 - 285 mµ to 275 - 280 mµ, and the concentration of estrogen could only be calculated accurately, after deduction of the absorption at 280mµ due to the impurities present. The application of this method to the estimation of small quantities of estrogens after hydrolysis could only succeed after elimination of the impurities. At the same time, however, it was established, that the absorption curve of the impurities is a straight line in the region of 310 mµ to 270 mµ, and in all determinations it was found, that the absorption due to the impurities at 280 mµ was 1.4 x the absorption at 310 mµ. As the estrogens do not absorb light of a wavelength of 310 mµ, it was found possible, to calculate the amount of estrogen present, by deduction of the absorption of the impurities at 280 mµ, (calculated as 1.4 x the absorption at 310 mµ).

All fractions, which had shown a maximum in the region of 280 mu, were tested with the Kober reagent, and it was found, that estrone fractions (λ_{max} 286 mµ) and estriol fractions (λ_{max} 275 and 280mµ) gave positive reactions, while all other fractions tested gave negative results. There are two possible interpretations of these finding — both probably true:— a) the Kober test is less specific than claimed. b) the estrogen spectrum was shifted by interfering substances from 280 to 286 and 275 mµ.

To remove interfering substances and to increase the quantity of estrogenic material present in urine, Smith and Smith's zinc and hydrochloric acid hydrolysis (234) of the urinary conjugates was chosen. Weak and strong phenolic fractions were obtained. On chromatography no estriol could be detected, but the presence of 800 µg estradiol was deduced from the extinction at λ_{max} 280 mµ in the "estradiol" eluate.

This investigation did not result in the discovery of a simple and reliable method for the estimation of small quantities of estrogens in urine, on account of the presence of phenolic interfering substances, which could not be eliminated. Another limitation — the non-specificity of the spectrophotometric method — was overcome. Separation of the estrogenic hormones and some of their products was accomplished by solvent partition, Girard's reaction and chromatography, mixtures of acetone and benzene being used as eluants.

3. Experimental Work.

1. The ultraviolet absorption spectrum and the molar extinction coefficient of progesterone.

Progesterone (0.6 mg.) was dissolved in ethanol (100 ml.), and the solution was analyzed spectrophotometrically. Readings were taken from 222 mμ to 290 mμ. At λ_{max} 240 mμ the molar extinction coefficient (E_m) was calculated.

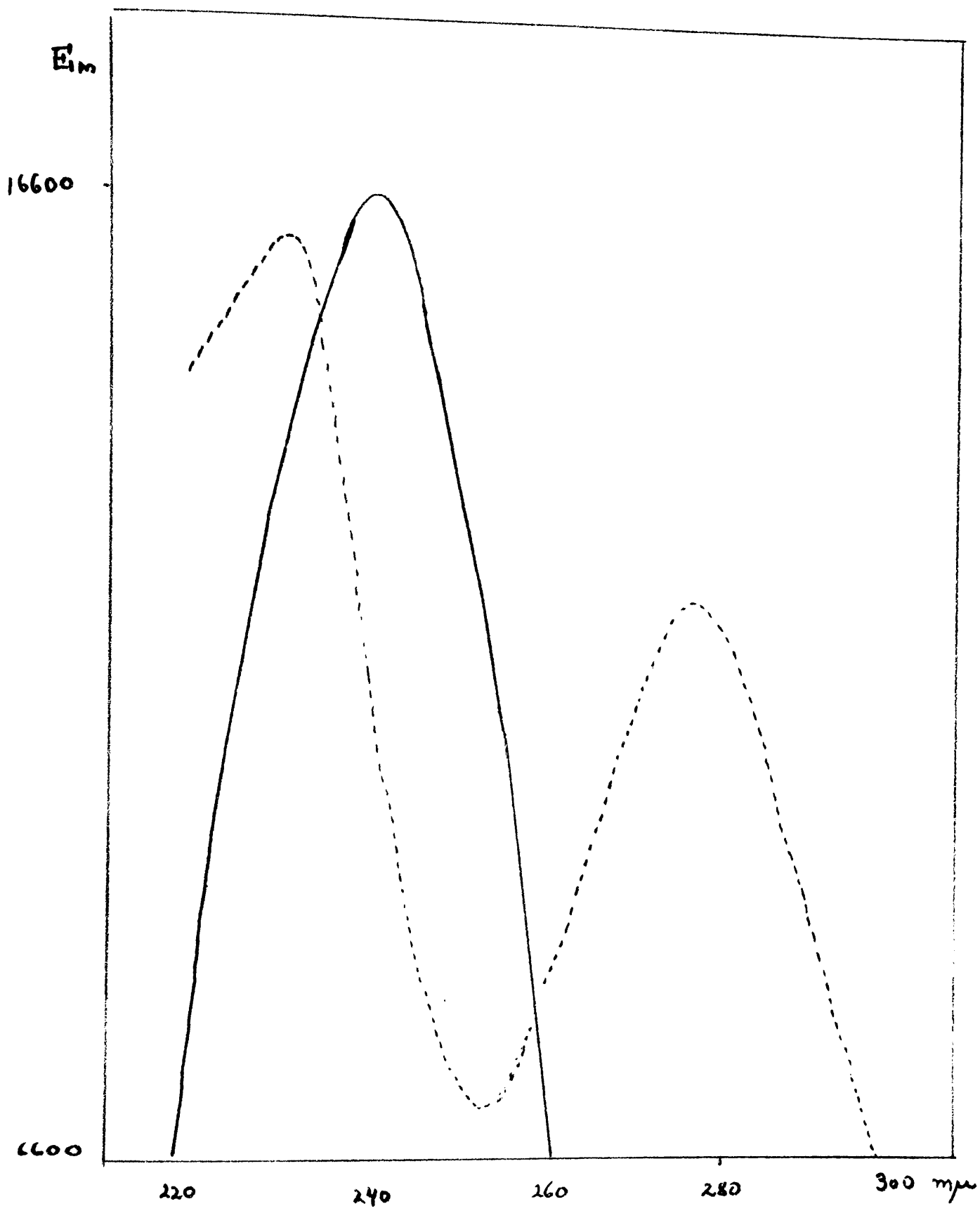
E_m = - (log Io/I x Molecular weight)/ concentration

= 16600.

2. The recovery of Δ4:5-cholestenone and progesterone added to dried plasma.

Δ4:5-cholestenone(51.6 mg)(CXII) was mixed with lyophilized plasma(65.6 mg.) and extracted with ethanol(6x25ml.). The solution was filtered and taken to dryness. The residue was dissolved in ether (50 ml.), washed with N sodium hydroxide solution (3x20 ml.), to remove acids and phenols, washed neutral with water, dried with sodium sulphate and taken to dryness. The residue was thoroughly desiccated. It was then treated with Girard's reagent "T", as described in part 1 B, section 6. The non-ketonic fraction consisted of 1.6 mg oil, while in the ketonic fraction 45.9 mg. crystalline material were isolated, which were identified as Δ4:5-cholestenone. 90% of the material had been recovered.

Absorption Spectra of Progesterone (——) and Ketonic Lipids of Blood Serum (----).



To determine the completeness of hydrolysis of the ketonic complexes in four hours, the aqueous phase was again extracted with ether after 20 hours. An additional 2.7% of 4:5-cholestenone was recovered. The non-ketonic fraction was again treated with Girard's reagent "T" without yielding ketonic material on hydrolysis.

The same procedure was followed in a recovery experiment on progesterone (4.1 mg.), added to dried plasma (54.7 mg.). 90% of the hormone was recovered in the ketonic fraction.

3. Chromatography of progesterone.

The oily residue, containing the recovered progesterone, was dissolved in benzene (1 ml.), diluted with 20 volumes petrol ether and adsorbed on a column of alumina. Crystalline progesterone was recovered on elution with 100% benzene and benzene:ether(20:1)mixture.

4. Examination of dried pooled blood plasma.

Dried plasma (770 mg.) was extracted with ethanol (4x30 ml.)(with stirring on the steam bath.), and the solutions were decanted. The residue was then extracted with a chloroform: ethanol mixture(1:1)(3x30 ml.). The latter extracts were not pooled with the ethanol extracts, but both were taken to dryness, dissolved in ether(30 ml.), washed with N sodium hydroxide solution (3x15 ml.), with water until

neutral, aried with sodium sulphate and taken to dryness. Both residues were separated into ketonic and non-ketonic fractions with Girard's reagent "T".

The chloroform, ethanol extracts yielded 1.5 mg. of ketonic material, which showed an absorption band only in the region of 275 mμ, and was discarded. The ethanol extracts gave 5.4 mg. ketonic and 27.6 mg. non-ketonic oils. The absorption spectrum of the ketonic fraction possessed two maxima: one at 275 mμ(simple ketones), the other at 230 mμ. (Graph: Page 106) No maximum at 240 mμ was observed.

5. Repetition of the extraction of blood plasma.

To investigate, whether reproduceable results could be obtained with the procedure of extraction and purification, three batches of 500 mg. of dried plasma were extracted, fractionated and analyzed spectrophotometrically. Identical spectra, with maxima at 230 and 275 mμ and similar intensity, were obtained.

6. The interference of the 230 mμ maximum with the detection of progesterone added to plasma.

Progesterone (0.5 mg.) was added to dried plasma (1 g.), and the mixture was extracted with ethanol, purified and fractionated in the usual manner. In its absorption spectrum, the ketonic residue showed a maximum at 236 mμ, indicating the presence of 1.336 mg. α,β-unsaturated ketones (calculated as

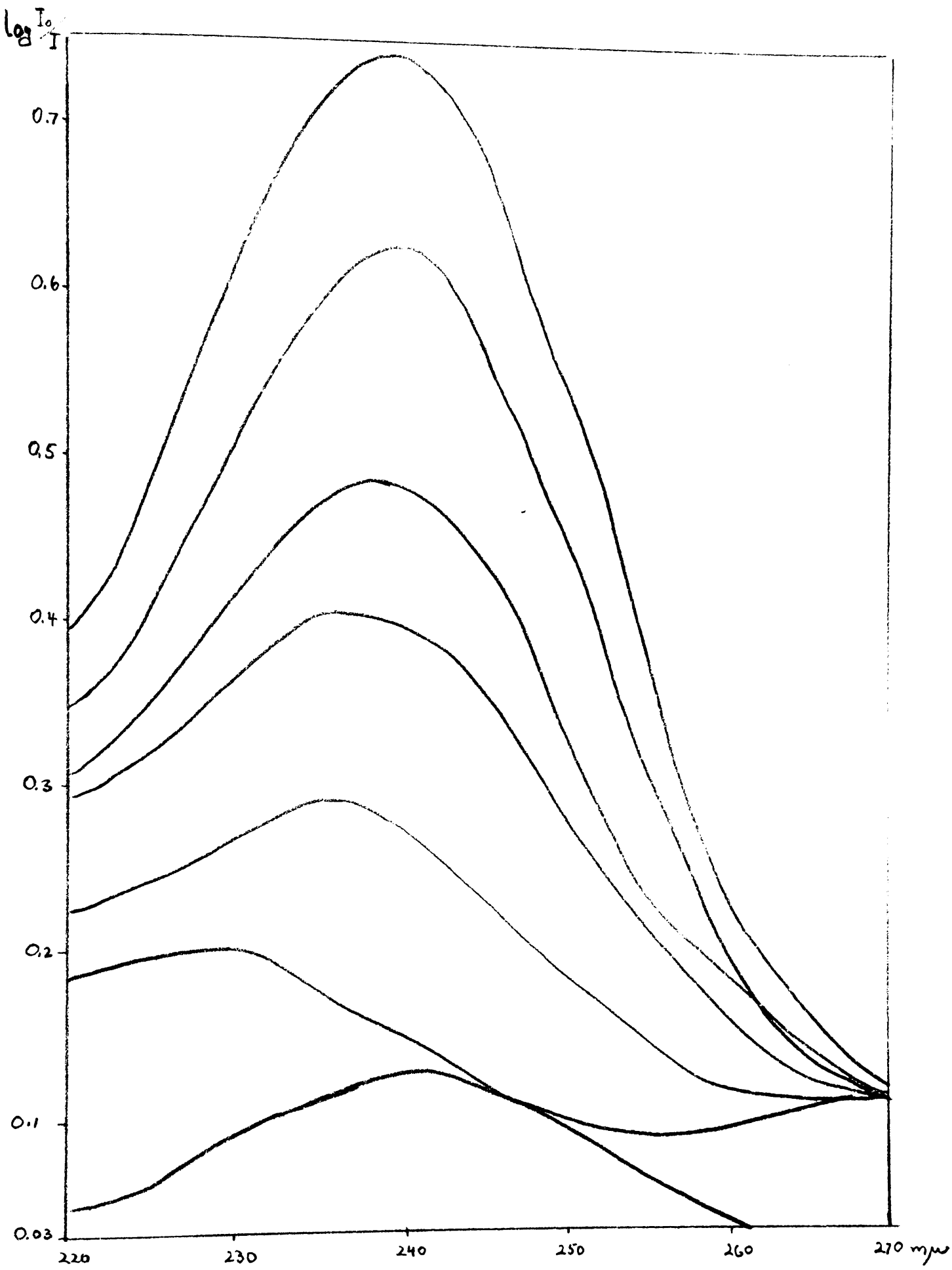
progesterone). The experiment was repeated with the addition of 2 mg. of progesterone to 1 g. dried plasma. A maximum at 240 mμ was observed and the presence of 2.83 mg. progesterone was deduced from the observed extinction. Both estimates were too high, and the absorption due to 830 μg. of "progesterone" per one g. dried plasma has to be attributed to the unidentified material (λ_{max} 230 mμ).

Another sample of dried plasma(350 mg.) was extracted and the ketonic fraction was dissolved in 100 ml. ethanol in a volumetric flask. The absorption maximum at 230 mμ was again observed. To this solution 1 ml. of a standard progesterone solution(226 μg.) was added, and the volume was reduced to 100 ml. by evaporation under a stream of nitrogen. The absorption spectrum of the solution was recorded (page 110.), and the solution was returned quantitatively to the volumetric flask. The addition of 1 ml. standard progesterone solution was repeated four times, and the solutions were analyzed spectrophotometrically. The results are given in a table.

ml. standard progesterone solution.	in 100 ml. solution.	λ_{max} (mμ)	μg. (calculated as progesterone)
1	ethanol	240	226
0	extract	230	295
1	"	232	515
2	"	236	757
3	"	238	953
4	"	240	1195
5	"	240	1401

It was concluded that in the presence of the interfering substance quantitative estimation of progesterone is impossible.

The Estimation of Progesterone in the Presence of the interfering Maximum at 230 mμ.



7. Chromatography of the neutral, ketonic extract of dried plasma.

The neutral, ketonic fraction (from one g. lyophilized pooled plasma) was dissolved in benzene (1 ml.), and petrol ether was added to incipient turbidity. The solution was poured on a column of alumina (Harshaw, acid washed, reactivated, petrol ether washed) and eluted with petrol ether : benzene and benzene : ether mixtures. The fractions were taken to dryness and analyzed spectrophotometrically. While no fraction showed a 240 mμ absorption band, a number exhibited maxima at 230 and 235 mμ. With 100% benzene saturated ketones were eluted (λ_{max} 274 mμ).

Attempts to remove the interfering substances by sublimation and solvent partition remained unsuccessful.

8. Lyophilisation of rabbit blood serum.

Blood (4ml.) was obtained from a rabbits ear vein. The serum (1.5 ml.) was spread over the whole inner surface of a test tube (with a ground glass joint and a connection to the high vacuum pump). The serum was thus frozen by immersion in a carbon dioxide snow - acetone mixture and when frozen, the test tube was attached to the high vacuum pump, where the ice sublimed without melting. Water vapour condensed to ice on the outside of the tube, and its melting was an indication that the sublimation

was ended. The vacuum was released, and the residue (117 mg.) was extracted with ethanol and purified as described previously. The small amount of ketonic material showed no maximum in the region of 240 mμ on spectrophotometric analysis.

9. Search for progesterone in the serum of women, seven days before menstruation.

One ml. samples of serum(from venous blood) were lyophilized, extracted and fractionated as described above. An absorption maximum was observed in all extracts at 230 or 252 mμ together with smaller maxima at 260 and 278 mμ. The extracts were chromatographed, but did not reveal the presence of a λ_{max} 240 mμ band.

10. Search for a 240 mμ absorption band in male serum.

Serum (2 ml.) from a venous blood sample was lyophilized, extracted and fractionated. On spectrophotometry, a number of maxima were observed at 222, 252, 264, 275 mμ. Approximately the same absorption spectrum was obtained from male and female serum extracts.

11. Introduction of a new extraction method for lipids from blood.

One ml. serum (from three ml. venous blood) was placed into a 25 ml. separatory funnel, to which 5 ml. ether were added. The mixture was shaken vigorously, and the emulsion

was frozen in a carbon dioxide snow-acetone mixture. The ether was decanted, fresh ether was added, and the ice was allowed to melt at room temperature. The emulisification and freezing process was repeated three times. The ether extracts were purified and fractionated in the usual manner. The ketonic fraction showed a maximum at 232 mμ besides the maximum at 278 mμ.

12. Comparison of the two extraction methods.

Aliquots of serum were extracted by a) lyophilisation and b) freezing in an emulsion with ether. Both extracts were fractionated in identical manner and their absorption spectra were found to be quantitatively and qualitatively similar. Both showed the 232 mμ absorption band.

13. Analysis of a blood plasma sample.

Two aliquots of plasma (1 ml.) from 5 ml. oxalated venous blood were extracted by method a) and b) respectively. On analysis of their spectrum no difference was observed . Both showed the 232 mμ absorption maximum.

14. Analysis of blood in advanced pregnancy.

Serum (1 ml. samples) was extracted by lyophilisation or freezing in emulsion with ether, purified and fractionated. The ketonic fractions showed absorption bands at 232 and 274mμ. It was concluded that the discovery of material, absorbing maximally at 240 mμ, in one ml. serum was impossible, since on chromatographic separation, the interfering substances could

not be completely isolated, to allow the detection of minute amounts of α,β-unsaturated ketones.

15. Examination of plasma and red blood cells for progesterone.

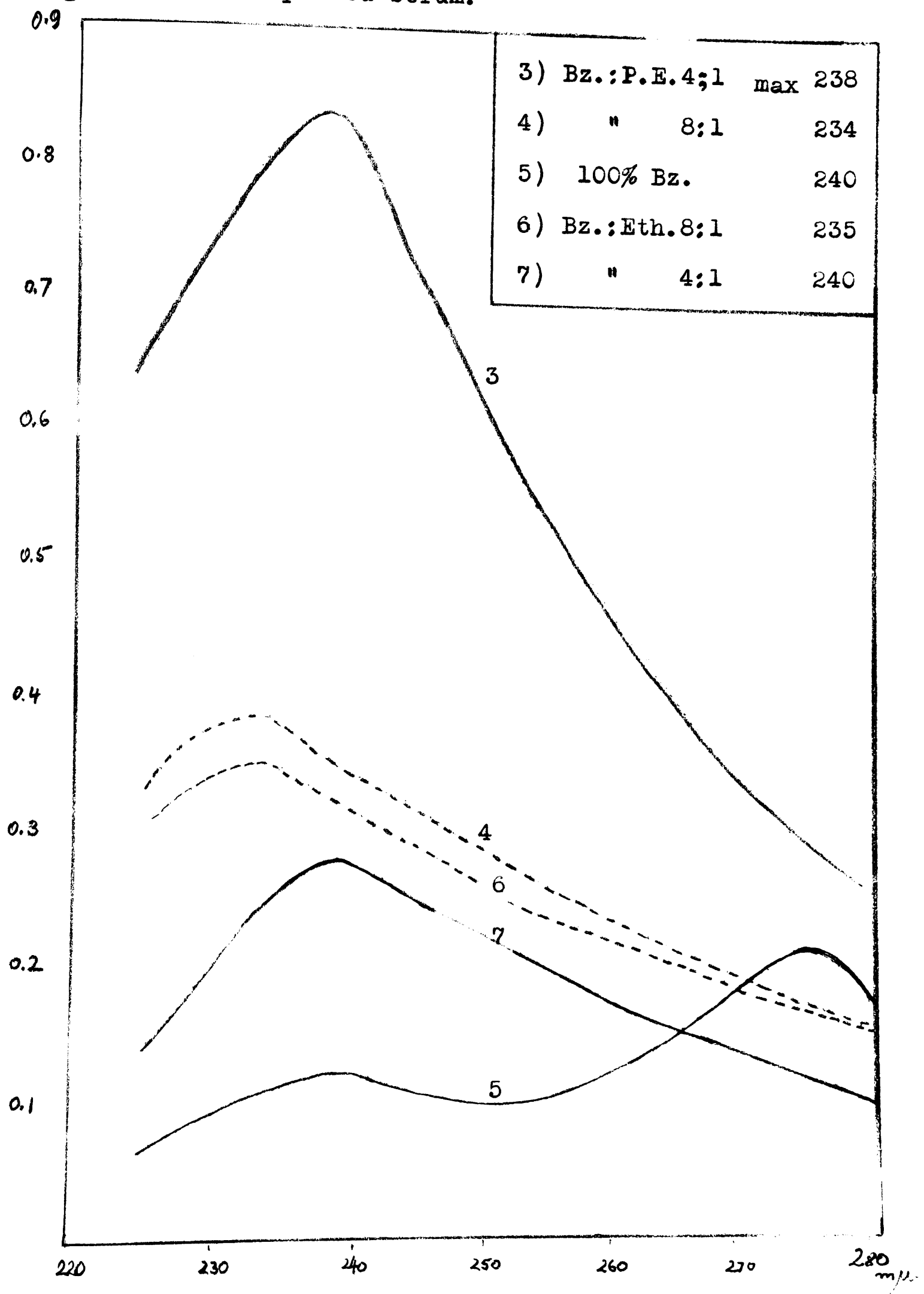
Two 5 ml. serum samples from women, seven days before menstruation, were lyophilized and fractionated. Only absorption bands at 230 and 278 mμ were observed.

The red blood cells from 20 ml. of blood were washed with saline and suspended in distilled water. The laked cells were then extracted by the ether emulsification and freezing method. The ketonic fraction possessed a maximum at 230 mμ, which reached a minimum at 225 mμ. No maximum was observed in the region of 275 mμ.

16. Analysis of a large sample of serum and extraction at pH 1.

On acidification of 100 ml. of serum (pooled) with concentrated hydrochloric acid, proteins precipitated. The mixture was extracted by the "ether-emulsion-freezing" method and the protein precipitate was reextracted with boiling ether. The combined extracts were fractionated. Besides the expected absorption maxima at 232 and 234 mμ, one band was observed at 238 mμ and two band at 240 mμ. Calculated on the basis of the molar extinction coefficient of progesterone, these bands indicated the presence of 47.7 μg "progesterone" in fraction 5, 7 μg. and 12.5 μg. "progesterone" in fraction 5 and 7. According to the elution of pure progesterone (section 3), fraction 5 must be assumed to represent the progesterone in 100 ml. pooled serum.

$\log \frac{I_o}{I}$ Absorption spectra of ketonic lipid fractions of 100 ml. pooled serum.



The Estimation of Estrogens in Non-Pregnancy Urines.

1. Hydrolysis, extraction and partition of urinary estrogens.

Samples of 24-hour specimens of urine were obtained from a woman on the 18th, 21st and 24th day of a normal menstrual cycle. The specimens were collected under toluene and stored in the refrigerator.

The urines were refluxed with 150 ml. of concentrated hydrochloric acid per liter of urine for ten minutes. They were rapidly cooled under a stream of cold water and the dark solutions were extracted with ether(3x1/3 volume).

The aqueous phase was run off, and the ether was filtered to remove solid matter and to break emulsion. The residue was thoroughly extracted with hot ether, which was added to the ether extract. The latter was then washed with 10% sodium carbonate solution (5x100 ml.), the aqueous phase being discarded. The ether was washed neutral with water and distilled. The oily residue was taken up in benzene (100 ml.) and extracted with 10% sodium carbonate solution(5x50 ml.). The aqueous phase was back-extracted with benzene(1x50 ml.), acidified and extracted with ether(5x50 ml.). The ethereal extracts were washed neutral and taken to dryness.("Strong phenols").

The benzene back-extraction was added to the original benzene solution, and the combined mixture was

extracted with 1 sodium hydroxide solution(3x30 ml.). The soda extracts were acidified and extracted with ether.(3x30 ml.) The ether solution was washed neutral with water and taken to dryness. The residue, the "weak phenols", was dried in a vacuum desiccator. It was then fractionated into a ketonic and non-ketonic fraction by an adaptation of the micro - Girard separation of Fincus and Pearlman(387).

Approximately 20 mg.Girard's reagent "T" (trimethyl acethydrazide ammonium chloride) and 0.5 ml. glacial acetic acid were added to the oily residue,"the weak phenols". The solution was then heated for 20 minutes under anhydrous conditions on the steam bath. The cooled solution was poured into 20 volumes of ice water containing sufficient sodium hydroxide solution (1 N) to neutralize the acetic acid used. The pH was checked and adjusted to 6.8 if necessary. The solution was then extracted with ether(3x15 ml.), and the total ethereal solution was washed with water, which was added to the aqueous phase, and taken to dryness."Non-ketonic weak phenols".

The ketonic complexes in the aqueous phase were hydrolyzed by the addition of concentrated hydrochloric acid to a concentration of 1 N. After four hours at room temperature, the liberated ketones were extracted with ether(3x25 ml.), the ether was washed neutral with water and distilled. "Ketonic weak phenols".

All three fractions were dissolved in ethanol (10 ml.), 0.2 ml. of the solution was removed by a pipette and diluted with 2 ml. ethanol. An absorption maximum at 280 mμ was not observed in any of the fractions.

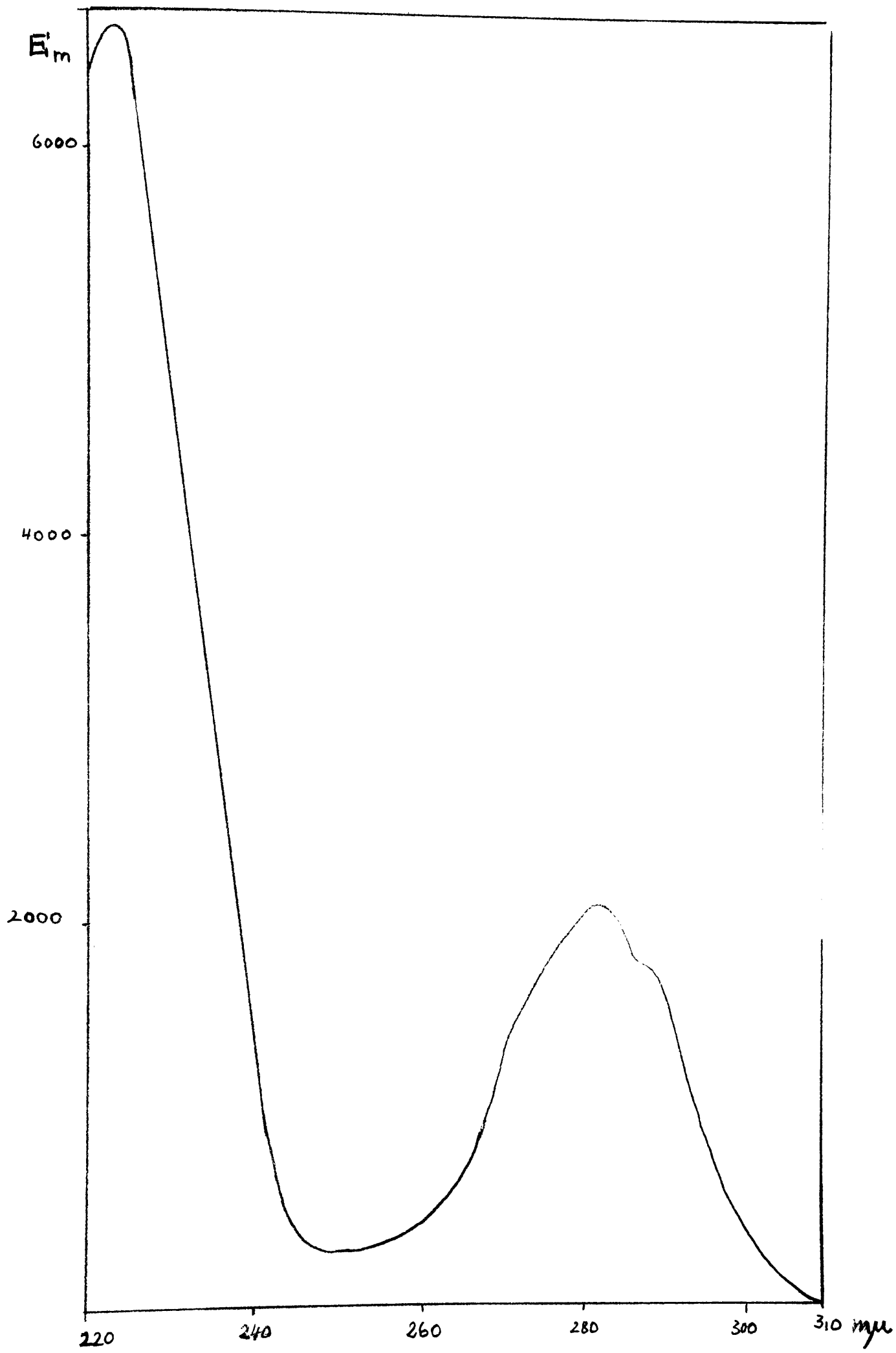
2. Chromatography of pure estrogens.

Samples of estrone, estriol and α-estradiol (2 mg.) were dissolved in a small volume of methanol, diluted with 40 volumes of petrol ether and separately chromatographed on columns of alumina. They were then eluted with 10 ml. portions of petrol ether containing increasing proportions of acetone. All eluates were taken to dryness and tested spectrophotometrically for the characteristic absorption band of the estrogens (λ_{max} 280 mμ). Estrone and α-estradiol were eluted with a mixture of 15% acetone in petrol ether, while estriol was obtained with 100% ether.

3. Chromatographic purification of urine extracts.

The oils obtained by partition of the urine extracts (section 1) were purified chromatographically. The "non-ketonic" phenols showed no absorption bands at 280 mμ in any of the eluates. The "ketonic" phenols contained a substance in their eluate with 15% acetone with an absorption maximum at 286 mμ, and the "strong" phenols showed a 280 mμ maximum in the 100% ether eluate.

The absorption spectrum of the estrogens and of sodium estriol glucuronate.



4. Purification by sublimation.

Pure estradiol, recovered from the chromatographic studies, was sublimed on the high vacuum at 115°C. The sublimate showed the typical absorption band of the estrogens, while the residue did not show a maximum. On sublimation of the urinary "non-ketonic, weak phenols", no trace of α-estradiol could be detected in the sublimate. On sublimation of the "strong phenolic" fraction at 115°C, a 280 mμ absorption band was detected in the sublimate (due to p-cresol probably), while the residue did not reveal the presence of estriol by its spectrum.

5. Repetitions with minor modifications of the fractionation of urinary estrogens.

Fresh 24-hour urine specimens were "hydrolyzed", heating the solutions uniformly in a glass-col heating element. The ether, used for extraction, was, shortly before use, shaken with ferrous sulphate solution (1%) (until the aqueous phase remained clear) and distilled. The ether extracts were quickly taken to dryness. No modifications of the solvent partition, Girard separation and chromatography were introduced. Similar results were obtained as in the first fractionation. α-estradiol could not be discovered in any of the urine extracts. The presence of estrone was indicated by the observation of λ_{max} 280 mμ in the 15% acetone eluate in one extract. Other extracts , including one male urine extract,

showed 286 mμ maxima in the 15% acetone eluates. The "strong phenols" revealed the presence of estriol in the 100% ether eluates in some of the extracts. No estriol was detected in the male urine extract.

6. The application of the Kober test to estrogenic fractions.

The Kober reagent was prepared by mixing equal weights of phenol and sulphuric acid, and heating the mixture for 15 minutes at 115°C. The solution was cooled and concentrated sulphuric acid (2 volumes) was added (310).

The test solution (1 ml.) was taken to dryness in a small pyrex test tube and the Kober reagent (1 ml.) was added. After heating the solution for 10 minutes on the steam bath, it was quickly cooled in ice water, concentrated sulphuric acid (1 ml.) was added, and the colour was observed. After further heating (½ minute) the colour was again noted. With hydrogen peroxide (0.5 ml.) the pink colour was discharged.

A number of fractions showing absorption bands in the region of 280 mμ, as previously described, were tested. The results are condensed in the table.

Fraction	λ_{max} mμ	description	Kober reaction
estrone	280	pure	positive
"	286	female urine	"
"	286	male urine	"
estriol	280	sublimed 115°	negative
"	275	" 180°	positive
estradiol	---	all fractions	negative

7. The absorption spectrum of the urinary impurities eluted with solvent mixtures of 15% acetone in petrol ether and the detection and estimation of estrogens added in small amounts.

A non-ketonic, weak phenolic fraction of a urine extract was chromatographed, and the "∝-estradiol" eluate (15% acetone in petrol ether) was taken to dryness, transferred to a 10 ml. volumetric flask and diluted to the mark with ethanol. The solution was examined spectrophotometrically. A "blank absorption curve" was thus obtained. The slope of the absorption curve was uniform from 310 to 260 mμ.

The solution was returned to the volumetric flask quantitatively, and 1 ml. of a solution of estradiol (containing 11 μg.) was added. The volume was reduced to 10 ml. and the absorption spectrum was examined. The process was repeated until 66 μg. of estradiol had been added. A 280 mμ maximum could not be detected. On calculation of the absorption at 280 mμ due to the estrogens added, correct recoveries were obtained, when the "blank absorption" was deducted.

The experiment was repeated on a new eluate with 15% acetone in petrol ether, and 60 to 120 μg. of estradiol were added. A maximum was observed at 280 - 285 mμ, which shifted with increasing amounts of estrogens to 275 mμ. The recovery was in good agreement with the amounts added, as long as the blank absorption was deducted. It was concluded from these observations, that the determination of small

amounts of estrogens in the presence of these impurities is impossible, but that in the case of larger quantities of estrogens, accurate determinations of their concentration can be made on deduction of 1.4 x the absorption at 310 mμ from the observed extinction at 280 mμ.

8. The elimination of impurities.

In an attempt to remove these interfering substances and to increase the quantities of estrogens extractable from urine, Smith's zinc and hydrochloric acid hydrolysis was chosen.(234)(with minor modifications.)

A 24-hour urine sample was autoclaved for 10 minutes with 15 volumes % hydrochloric acid, cooled and extracted with ether, and the aqueous phase was again autoclaved with 4% zinc for three hours. After cooling, the colourless solution was extracted with ether. The ether extracts of the two hydrolysates were combined. The solution was partitioned, separated by Girard's reagent, decolorized with zinc and hydrochloric acid and chromatographed. Impurities had been removed to a considerable extent. No "estriol" could be discovered, but the ketonic and non-ketonic 15%-acetone-eluates showed absorption maxima at 280 mμ. From the extinction at λ_{max} the presence of 800 μg. estrogen per 24-hour urine sample was deduced, representing three times the normal value at that time of the menstrual cycle.

α-estradiol into experimental animals, and isolation and identification of the urinary transformation products.

It was established, that the conversion of α-estradiol to estrone in vivo was a reversible reaction (12-18), and that both compounds give rise to estriol in the human(19-25). On administration of estriol, no other estrogenic compound could be isolated(26). Experiments on rabbits led to the interesting discovery, that α-estradiol and estrone are converted to β-estradiol (CXVII) only(27-31), and this substance proved to be considerably less active than any of the other estrogens. (267,268).

The conclusion was reached, that the organism is capable of transforming the most active estrogen into less active products by conversion, and that these products are then conjugated,making them hydrophilic for easier elimination by the kidney.(153,158).

In all experiments of this nature, the recovery of estrogens accounted for only 15% of the material given (32,33). The missing 85% were assumed to undergo chemical alteration to such an extent,that the steroid nucleus is broken up and physiological potency is lost.

The liver was established as the site of estrogen inactivation (34-72). Implantation of ovarian tissue and injection of estrogens into different organs of intact animals (34-52),perfusion experiments (53-55), liver slice (56-60)

and liver extract incubation with estrogens (61,62,43), and experiments on animals with liver damage(63-72) tended to show, at first glance, the loss of estrogenic activity through oxidative destruction by thermo-labile, cyanide-sensitive liver enzymes(58,73). The only other organ capable of inactivating estrogens is the kidney (74,75,45). The spleen was found to inactivate estrogens only, if it was drained into the liver(39,46-51).On transplantation the spleen lost its inactivating capacity.(46).

Further evidence for the oxidative destruction of estrogens in vivo seemed to come from the study of deficiencies of the vitamin B complex (76-92). Thiamine and Riboflavine appeared essential for the inactivation of estrogens.

Attempts to isolate the oxidative enzyme were made by Zondek and Pincus (93-105). Enzyme extracts were obtained from liver(61,62,98) and a variety of plant sources, which could oxidize estrogens. The enzymes were polyphenoloxidases and thus produced insoluble and sometimes coloured end products, making this type of inactivation physiologically improbable. (CXIV - CXXV - CXXVI).

A different pathway of oxidative destruction of estrogens was suggested by Westerfeld, who obtained a lactone by oxidation of estrone in vitro with alkaline hyarogen peroxide(106-108). Westerfeld's lactone is prepared by the open-

ing of ring D of the steroid nucleus.(CXVIII - CXX - CXXI). As the action of hydrogen peroxide in vitro resembles, in many cases, reactions occurring in the body, attempts were made to isolate the lactone or its hydrolyzed form, the hydroxy acid.(109). No trace of this compound could, however, be discovered, even after administration of a large dose of estrogens, in the urine. Smith and Smith(110,111) obtained evidence, that a non-estrogenic compound is present in urine, which on heating with zinc and hydrochloric acid shows estrogenic activity. It was assumed, that a compound, like Westerfeld's lactone was reduced to an active estrogen. On reduction of the first sample of Westerfeld's lactone, they succeeded in augmenting its estrogenic activity, but were unable to reproduce these results with purer samples. (112-114).

Smith and Smith attributed an important physiological role to their hypothetical oxidation product. As the ratio of inactive estrogen oxidation product to active estrogen (TZn/T_o) was greatest in the follicular phase of the menstrual cycle, the following hypothesis was advanced.

The inactive estrogen oxidation product stimulates the hypophysis to release its luteonizing hormone (L.H.), causing the formation of a corpus luteum. In the presence of progesterone, produced by the corpus luteum, the inactivation of estrogens by conversion to less active forms is facilitated, so that little of the inactive oxidation product is formed.

In consequence, pituitary stimulation subsides, the formation of luteonizing hormone ceases, and the corpus luteum degenerates. At this stage estrogen metabolism returns to the production of the inactive oxidation product and the sequence of events is repeated.

Evidence for this scheme, explaining some of the cyclic menstrual changes, is based on Smith's discovery, that Westerfeld's lactone possesses pituitary stimulating activity (112-114), and that estrogen conversion to estriol is facilitated in the presence of an active corpus luteum(22-24). Confirmation of this work came from Figge(115), who observed a similar pituitary-stimulating effect of estrone, which had been inactivated by light. It appeared, therefore, highly desirable to identify Figge's estrone inactivation product and Smith's urinary inactive oxidation product with Westerfeld's lactone.

Two other compounds were suggested as the inactive estrogen oxidation product. Marrian(116) obtained evidence, that estriol was oxidized to 16-keto-estrone (CXXIII)(the compound assumed to give the pink colour in the Kober test), which, on reduction with zinc and hydrochloric acid, gave rise to active estriol. The other compound, 6-keto-estrone was suggested by Gallagher(personal communication) as an artifact,(similar to the formation of 7-keto-cholesterol(212,213) during extraction)which on reduction with zinc and hydrochloric acid was expected to yield active estrogens.

Great doubts were cast on the complex mechanism of oxidative estrogen inactivation by the work of Cantarov et al. (117-134). They showed that the liver removes estrogens from the blood stream, excreting them into the bile. The estrogens pass with the bile into the intestine, where they are reabsorbed, and travel again to the liver. An entero-hepatic circulation of the estrogens is thus established. Cantarov's theory can explain the majority of experimental results on the loss of estrogenic activity. Experiments on tissue slices, tissue extracts and enzyme preparations , however, require the admission, that destruction of estrogens by oxidation in the liver can occur. It does not prove, however, the occurrence of oxidative inactivation at physiological levels and normal conditions. Polyphenolase activity of the extracts could explain the destruction, eliminating it as a physiological pathway.

The importance of the Vitamin B complex in the destruction of estrogens pointed to enzymatic dehydrogenation and decarboxylation of the estrogens. The lowered estrogen inactivation by liver tissue, deficient in methionine, seemed to involve a number of other processes in the oxidative destruction of estrogens. The inefficient estrogen inactivation of the liver under these conditions could be attributed to general inanition. (84, 87-90). Administration of the vitamins and methionine alone, could not reestablish efficient estrogen inactivation in a starved animal. (84). The entero-hepatic cir-

culation is impaired under this condition.

If the estrogens are not lost by oxidation in the liver, but pass into the entero-hepatic circulation, one cannot account for their destruction, unless it occurs in the intestinal tract. The faeces have been repeatedly examined for the presence of estrogens(73,138-139), but only small quantities of them were discovered. A study of the destruction of estrogens by bacteria was made by Zondek (140), who tested a variety of bacteria in pure cultures. Only two were found to destroy estrogens and both, a type of Proteus and B. mesentericus, are atypical for faecal flora.

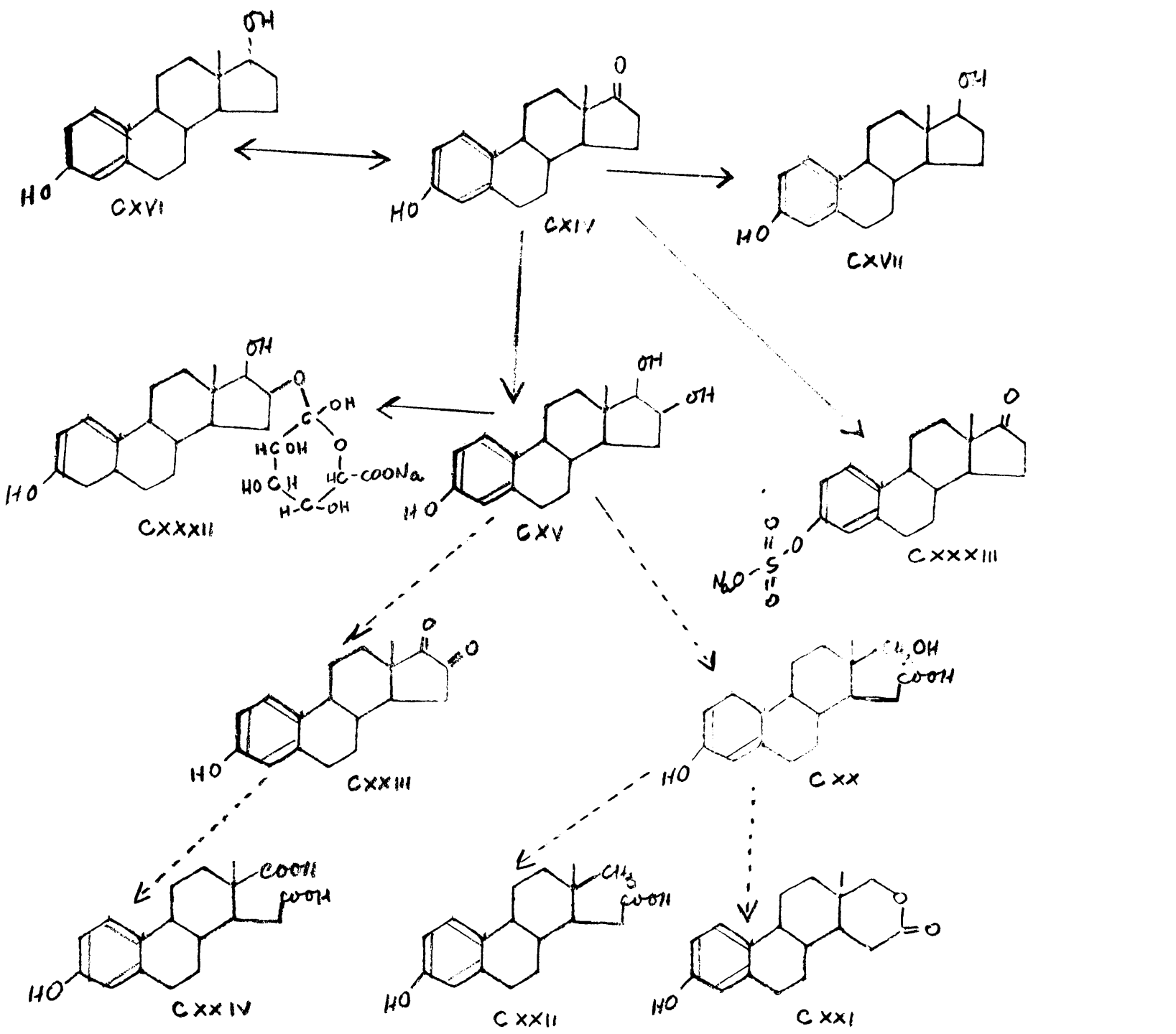
It is possible, that inactivation of estrogens by bacteria involves the reduction of the phenolic ring A to estrane-diols, which have been isolated from urines of normal women by Marker(141), (CXXVIII). Further evidence for such a reaction was obtained by Heard(142,143), when he isolated 5,7,9-estratriene-3-(β)-ol-17-one(CXXXI) from equine pregnancy urine.

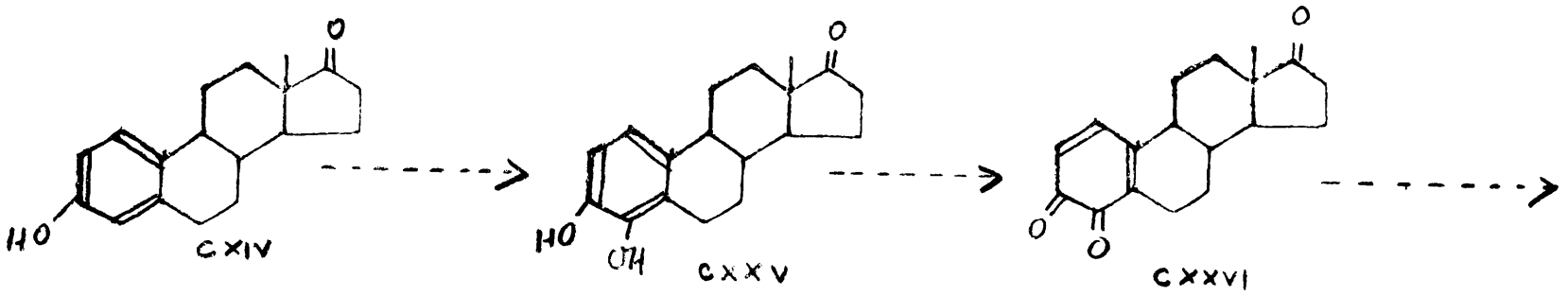
As the evidence for oxidative destruction of the estrogens decreased, attention was turned to the process of conjugation. The estrogens in blood were found to be in a water soluble form(145), and conjugation was postulated to occur in other organs besides the kidneys. Fishman(146-149) discovered that the activity of β-glucuronidase of liver and kidney could be increased by glucurogenic substances.

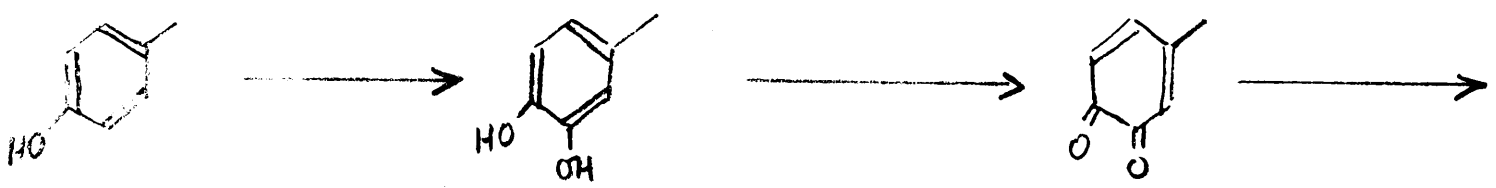
Glucuronidase activity could not be increased by these substances in the organs of reproduction. Estrogens, on the other hand, were able to augment the ß-glucuronidase activity of these organs. Their preferential up-take of estrogens had been established by experiments with synthetic estrogens, containing radio-bromine.(150).

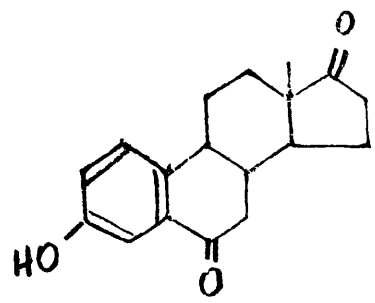
During pregnancy, the urinary estrogens are nearly all conjugated (except shortly before parturition). Sodium estriol glucuronate (CXXXII)(151-156) and estrone sulphate (CXXXIII)(158-163) have so far been identified. Both compounds are the products of an efficient detoxification process, as they possess low biological activity and high water solubility.

Prolongued circulation of the estrogens, preferential retention and interconversion in the organs of reproduction, followed by conjugation and, finally, excretion through the kidney are the only experimentally established facts, regarding the fate of the estrogens in vivo.

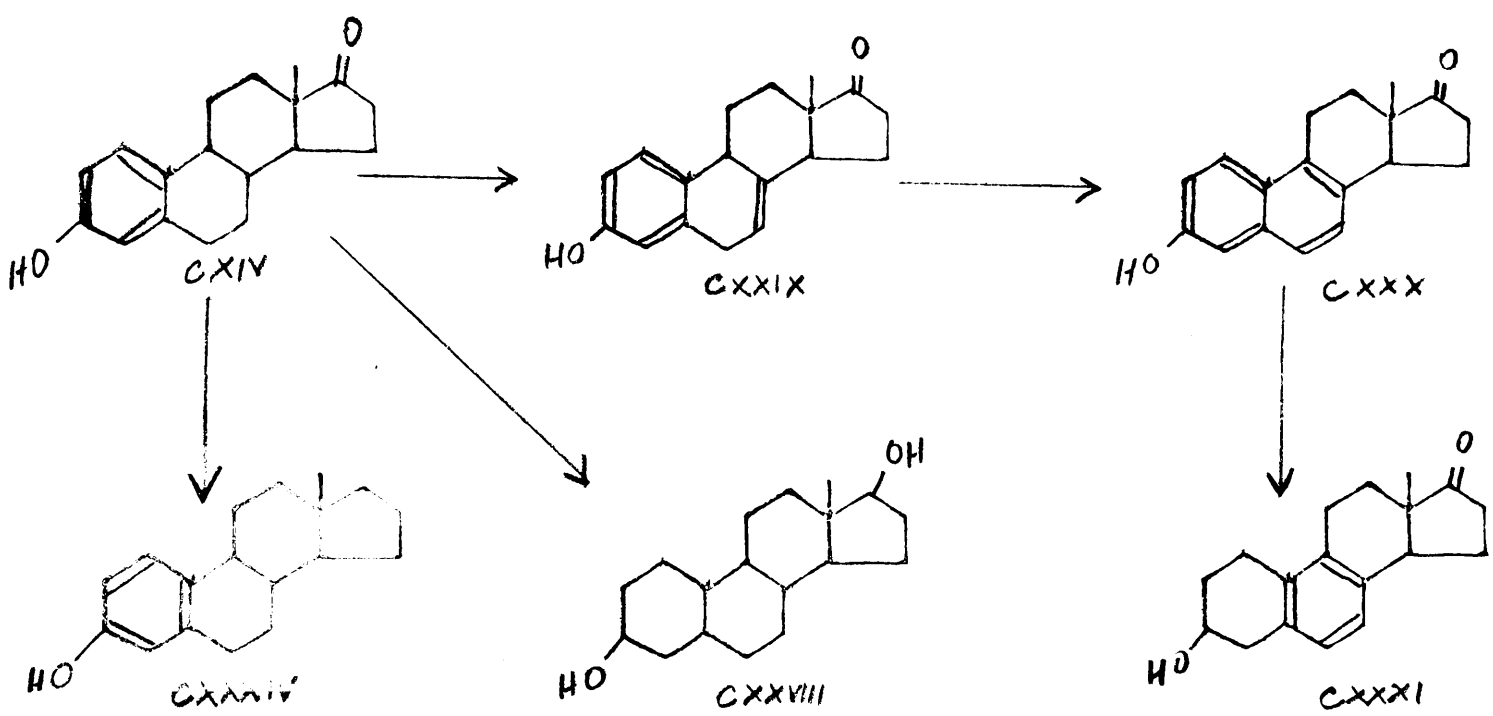

OH
HO
CXVI
O
CXV
CXVII
CXXXII
HC OH
HO C H
HC-COONa
H-C-OH
CXXXIII
Na O
CXXIII
COOH
CXXIV
CXXII
CH3
CXX
CXXI


CXIV
CXXV
CXXVI





CXXVII



CXIV CXXIX CXXX CXXXIV CXXVIII CXXXI

2. Discussion.

This investigation was undertaken, to confirm the findings of Smith and Smith by isolation and chemical identification of their hypothetical estrogen oxidation product, which could be reactivated with zinc and hydrochloric acid. The successful identification of the compound would not only place their application of these discoveries to clinical medicine on a firmer basis, but might also further elucidate the fate of estrogens, particularly that portion of the injected estrogens, which could not be recovered.

To obtain comparable results, their procedure was followed through hydrolysis and extraction with only minor alterations. For the separation of "strong" and "weak" phenols the Smiths used Marrian's method of partition(182). That method has been found much less accurate than the one of Bachmann(185) and Mather(186), used in all separations herewith described. Girard's separation of ketonic from non-ketonic material was used in both instances. Only minor changes were introduced. Instead of biological assay, the fractions were chromatographed and examined spectrophotometrically for the absorption spectrum, typical of the estrogens, (λ_{max} 280 mμ.). Also, Smith's hydrolysis with and without zinc was carried out on aliquots of urine, while in this work the two hydrolytic treatments were carried out on the same sample of urine.

A 2-liter urine sample of the 6th month of gestation served as starting material. On treatment"A"(10 minutes refluxing with 15 volumes % concentrated hydrochloric acid) ten mg. crystalline estriol was isolated. A similar quantity of estriol was obtained after treatment"B"(3 hours refluxing with 15 volumes % concentrated hydrochloric acid and 4% zinc dust). Crystals of estrone were obtained after each hydrolytic treatment in minute amounts, while estradiol could only be detected spectrophotometrically in both extracts.

As the presence of benzoic acid (λ_{max} 272 and 280mμ) interfered with the detection and estimation of estrogens, its complete removal by washing with 10% sodium carbonate solution was investigated. On examination of the soda extracts, it was found that 10% of the estriol was removed in a single carbonate washing. A thorough washing with 10% sodium carbonate solution was therefore impossible. A 9% sodium bicarbonate solution was tested by Bachmann and found, not to remove any of the estriol. It was found to remove benzoic and salicylic acid quantitatively, but many coloured impurities are not extracted.

Another preliminary investigation concerned the removal of estrogens from ether by extraction with N sodium hydroxide solution. Complete removal of all material showing a 280 mμ maximum was not possible, confirming Friedgood's observation. (personal communication).

A thorough investigation of a 4-liter urine batch (8th month of gestation) led to the isolation of all the known common urinary estrogens in pure crystalline form. Their melting points were taken, and they were quantitatively analyzed by spectrophotometer and weighed. The two determinations agreed. 42 mg. estriol, 1.8 mg estrone, 1.38 mg β-estradiol and 7.25 mg α-estradiol (only 3.46 mg was crystalline) were isolated.

Stimmel published in 1945 a simplified chromatographic separation of the estrogens (205-207), which was adopted for further investigations. Although it will not separate α and β-estradiol, the use of methanol instead of acetone will prevent any secondary reactions affecting the estriol present.

As the estrogen inactivation product, postulated by Smith, is supposed to be of the nature of Westerfeld's lactone, a search for the lactone was again undertaken. No trace of the compound could be detected. A search for the compound, with the lactone ring opened, was made in the sodium bicarbonate washings. They were methylated with diazomethane and chromatographed. None of the fractions showed a 280 mμ maximum.

From this chromatogram an oily fraction was obtained showing an absorption band at 256 mμ. It was suspected, that the band was due to an artifact, produced by

oxidation of the estrogens during hydrolysis and extraction. A similar absorption spectrum is shown by 6-keto-estradiol, which was prepared by Wintersteiner(211): λ_{max} 258 mμ. Reduction of this compound with zinc and hydrochloric acid would lead to estradiol. The small quantity of material could, however, not account for the great increase in activity.

A search for 16-keto-estrone also proved futile. This compound had been suggested by Marrian(116) to be the inactive oxidation product and had been synthesized by Huffman(215) from estrone. Its properties were described.

As large quantities of estriol were still isolated on treatment"B", protection of the estrogens from atmospheric oxygen was not the sole function of the hydrogen atmosphere. Whether zinc would protect the estrogens from partial destruction during treatment "A", was next to be investigated. One aliquot of urine(6th month of gestation) was treated in the usual manner ("A" and "B"), while the other was given treatment "B" only. Instead of the somewhat larger yield of estrogens expected in the latter, only half the amount of estrogens was recovered, compared to the combined yields of the first aliquot. This unexpected observation was made in all three estrogenic fractions, although only in the estriol fraction could it be established beyond doubt by isolation of crystalline estriol.

As these results were reproducible, the comparison of the two methods of hydrolysis was further studied. One aliquot of pregnancy urine was given treatment "A" and "B", while the other was refluxed for 10 minutes with 15 volumes % concentrated hydrochloric acid and 4% zinc dust and, after extraction, was given treatment "B". The yields of estrogen (crystalline estriol) was 7.5 mg. and 13 mg. in the first aliquot and 4.5 mg and 17.6 mg. in the latter.

In the light of these observations two explanations can be advanced: Zinc retains a large proportion of the estrogens by adsorption and secondly, the addition of zinc lowers the pH of the solution so rapidly, that hydrolysis will only be taking place part of the time. The final pH after treatment "B" was found to be 3.5-4.5, at which according to Schmulowitz (237) no hydrolysis of the estrogen conjugates occurs.

This interpretation of the results obtained led to a new approach towards the solution of the problem. Since the days of Laqueur's discovery, that the estrogenic activity in urine could be greatly increased by acid hydrolysis, a variety of methods for the liberation of urinary estrogens has been published. (219-250, 255, 256). No agreement was reached as to the method, which gave maximum yield, as two reactions proceed simultaneously, i.e. hydrolysis of the estrogen conjugates and destruction of the liberated estrogens. (252-254, 166).

Smith and Smith had accepted, that the method adopted by them gave maximum yields, and that no further hydrolysis would be expected to occur. They, therefore, assumed, that reactions other than continued hydrolysis of the conjugates took place. While it was certain, that hydrolysis of estriol glucuronate would not occur to any extent at room temperature in the presence of zinc and hydrochloric acid, reduction of the unconjugated, water soluble estrogen oxidation product should take place, as judged from the vigorous evolution of hydrogen.

Aliquots of pregnancy urine were given treatment "A". One was then allowed to stand at room temperature for 16 hours in the presence of 15 volumes % concentrated hydrochloric acid and 4% zinc dust. It was extracted and given treatment "B". The other aliquot was given treatment "B" right after "A". While estrogens were isolated after treatment "B", not a trace of estrogenic material could be discovered after reduction at room temperature. Reduction by itself cannot be held responsible for the increase in estrogenic activity. (Spectrophotometric analysis does not distinguish between estrone and Δ-estradiol and no increase in estrogen activity is recorded on reduction of estrone to estradiol.) If an estrogen oxidation product is present, it cannot be ether-soluble, nor readily hydrolyzed or reduced, but must be stable during acid hydrolysis in the presence of oxygen.

On chromatographic separation, it had been observed, that a large quantity of material was not eluted, but remained on the column after repeated washing with methanol. Before any conclusions regarding the existence of an estrogen inactivation product could be reached, this fraction had to be investigated. The material was eluted with N sodium hydroxide solution, extracted, after acidification, with ether and taken to dryness. Two crystalline compounds were obtained. One was very soluble in petrol ether and showed the absorption spectrum of benzoic acid, but differed in a number of properties. It gives a purple colour with ferric chloride, is only eluable with sodium hydroxide from alumina, and has a lower melting point. The empirical formula, $C_7H_6O_3$, suggests a hydroxy-benzoic acid (meta or para).

The petrol ether insoluble crystals were rechromatographed on a column of celite and magnesium silicate, and crystals were eluted possessing an absorption spectrum similar to that of salicylic acid, gave the same colour reaction with ferric chloride, but differed in its melting point. (183-184°C) As only small amounts of these compounds were isolated, attempts were made to isolate the materials from subsequent urine extracts. They could, however, not be obtained, although identical treatment of the urine was used.

The remaining oily fractions did not show the 280 mμ absorption band and it could be concluded that the isolation of the oxidation product of estrogens had not succeeded.

The nature of the estrogen precursors after treatment"A" and before "B" was investigated. The estrogen conjugates were extracted with n-butanol, and the n-butanol extracts were washed with N sodium hydroxide solution. The aqueous phase was not expected to contain any of the known conjugates, while the butanol should contain estrone sulphate and the soda washings all estriol glucuronate. The estrogens in all three fractions were freed, and from the results neither the existence of an estrogen inactivation product conjugate nor the presence of the known estrogens in a different conjugation could be deduced.

Partition of the urinary estrogen conjugates, before any hydrolytic treatment, was undertaken, to investigate the existing conjugates and their rates of hydrolysis. The urine was acidified to pH 3, extracted with n-butanol, which was washed with N sodium hydroxide solution. The butanol extract was washed, condensed and diluted with 20 volumes water. The soda extract was neutralized and all three solutions were given treatment "A", followed by "B". An aliquot of urine was used as control, receiving treatment "A" and "B" without partition. All estriol was obtained on treatment "A" from the soda extract, where it was expected to be. However, it was surprising to find complete hydrolysis of the estriol glucuronate in distilled water on treatment "A", while in urine the same amount of estriol was only obtained after both hydrolytic treatments. From butanol the estrone could be obtained completely on 10 minutes hydrolysis. Only small amounts of estrogens (10%) were isolated from the aqueous phase.

The evidence of all these experiments pointed to incomplete hydrolysis, and the action of zinc was reduced to a role of protecting the liberated estrogens from destruction by atmospheric oxygen, besides the role of reducing estrone and thus increasing its potency on bio-assay.

To test this hypothesis, aliquots of pregnancy urine were autoclaved for three hours at 120°C and 15 pounds pressure in the presence of 15 volumes % hydrochloric acid with or without 4% zinc. Approximately equal amounts of estrogens were isolated in both cases.

The validity of the assumption put forward was further put to the test by a number of hydrolytic treatments of urine aliquots, either in air or in the autoclave, with or without zinc and, in one case, in an inert atmosphere of nitrogen. The lowest yield of estrogens was obtained on hydrolysis first for 10 minutes and then for 3 hours in air without zinc. The yield was practically the same for all others. Less than 50% of the total estrogens were liberated during 10-minutes hydrolysis.

In an attempt to determine the length of treatment required for complete hydrolysis, it was observed, that the aliquot, receiving 10-minute treatments at a time, yielded much less estrogens than the one which had been hydrolyzed for the same length of time without interruption. Only 40% was obtained from the first aliquot. A similar observation

had been made by Marrian(230), that admission of air lowered considerably the yield of estrogen on hydrolysis.

The experimental results pointed without exception to incomplete hydrolysis of the conjugates. Additional evidence came from the observations of Marrian(156), who obtained 80% of the estriol present on hydrolysis of pure sodium estriol glucuronate in the autoclave at pH 1 for two hours. In urine the liberation of the estrogens proceeds more slowly and destruction of the free estrogens also occurs less rapidly, (166). Keeping these facts in mind, the hydrolysis of sodium estriol glucuronate and the destruction of estriol were studied on model experiments.

Impure sodium estriol glucuronate, which analyzed spectrophotometrically 11% estriol, was purified, until the sodium estriol glucuronate content was raised to 80%. The hydrolysis of the conjugate was then studied through the application of spectrophotometric analysis to the determination of the recovered estriol.

Hydrolysis in the autoclave(120°C, 15 pounds) at pH 1 was studied first. During 10 minutes no hydrolysis occurred. After 3 hours in the presence of zinc, 82% estriol was extracted and after readjustment of the pH from 3.5 to 1, one-hour hydrolysis brought the total recovery to 94%.

To compare the rate of hydrolysis and destruction,

four aliqots of estriol glucuronate were hydrolyzed under identical conditions(autoclave, pH 1) with and without zinc. Two were extracted after every 10-minute hydrolysis, while the other two were only extracted after the last period of heating. The recovery of estriol amounted to 90 - 100% in all four aliquots.

Hydrolysis of the conjugate with zinc in the presence of atmospheric oxygen yielded 75% of the estriol after a 2-hour treatment. In the absence of zinc the recovery, under identical conditions, only amounted to 42%. None of the unaccounted 58% could be recovered on subsequent hydrolysis. A yellow oily residue was obtained, which did not show the characteristic spectrum of the estrogens. Destruction of estriol on hydrolysis in the presence of air was considerable.

Smith's method of hydrolysis was next tested on estriol glucuronate. The solution was acidified to the pH, obtained on addition of 15 volumes % concentrated hydrochloric acid to urine. After refluxing for 10 minutes, 55% of the conjugate had been hydrolyzed and after 16 hours at room temperature, an additional 5% free estriol could be extracted. The remaining 40% were recovered on extracting the aqueous phase with n-butanol. Destruction of estriol had not occurred, but hydrolysis of the conjugate had not been complete.

Smith had accepted treatment"A" as an efficient method of hydrolysis and had concluded, that additional estrogens, obtainable on prolongued hydrolysis with zinc, could not be explained on the basis of incomplete hydrolysis, because on continued hydrolysis without zinc no further yields of estrogens were obtained. This rapid destruction remained to be explained.

A comparative study of the majority of methods for the hydrolysis of estrogen conjugates was undertaken. Marrian's method(156) gave quantitative yields. With Smith's directions 70 - 80% could at best be recovered. In the presence of zinc the yield was found to be even lower. The remaining methods tested gave yields ranging from 0 to 94% and were only of interest for the study of their experimental results.

When treatment "A" was followed by treatment"B", good recoveries were obtained, while "B" alone gave poor yields. On hydrolysis after "A" without the addition of zinc no estriol was obtained and 40% of the estriol was destroyed. On the other hand prolongation of treatment "A" beyond 10 minutes did not produce yellow oily material devoid of a 280 mμ maximum, although the yield of estriol was only little improved. A second hydrolysis with zinc yielded additional 20% of the estriol.

An explanation of these results could only be found in the presence of ether, dissolved in the aqueous phase, which in the presence of air would form ether peroxides, accounting thus for the destruction of estriol. The action of zinc would be instrumental in preventing the formation of ether peroxides by an effective hydrogen atmosphere. The theory was tested, estriol was dissolved in water and refluxed with 10 volumes % hyarochloric acid in the presence of ether. No trace of estriol could be recovered.

In the presence of zinc only 50% were recovered. As this loss of material had been observed on hydrolysis of the estrogen conjugates of urine in the presence of zinc, it was further investigated. Estriol was dissolved in water and its concentration was determined spectrophotometrically. Zinc was added and the solution of estriol was shaken. The suspension of zinc was removed by filtration, and the solution was found to contain the same amount of estriol as before. Zinc was added again to the solution and acidified. It precipitated and hydrogen gas was evolved. The supernatant solution contained only 80% of the estriol added. On extraction with ether, only 80% were recovered.

To explain this loss of estriol (suspected to be an adsorption phenomenon), estriol glucuronate was hydrolyzed in the presence of varying amounts of zinc. The solution, in which all the zinc had been dissolved, gave quantitative yields on hydrolysis. In the presence of residual zinc only

75 - 83% yields were obtained after treatment "A" and "B". On very thorough extraction, estriol can be recovered from zinc.

The hydrolysis of estrone sulphate was then investigated. 10-minute hydrolysis (10%HCl) was found sufficient to free all the estrone. As weak phenols were obtained after the second hydrolytic treatment of urines, delayed hydrolysis, due to the buffering effect of urine, was postulated. The possibility of a different type of estrone conjugate could not be rejected.

In their investigation Smith and Smith had found, that on zinc hydrolysis the activity of estrone was increased 5 times (bioassay on ovariectomized rat) and they accepted this factor for all hydrolyses in the presence of zinc. On the reduction of estrone in vitro (257-270), 60 - 80% was found to be α-estradiol, and the rest was ß-estradiol. As the activity of α-estradiol : ß-estradiol : estrone is given as 12 : 0.3 : 1 by Wintersteiner (267) and as 8 : 0.08 : 1 by Butenandt (268), standardisation of the conditions of reduction seemed of utmost importance. Small variations in the proportions of the α:ß-form will cause great errors on bioassay. The action of zinc and hydrochloric acid on estrone was therefore studied.

When three mg. of estrone were reduced by Smith's method of hydrolysis, 90% was recovered as desoxyestrone and

10% as estradiol, of which the greater part was identified as α-estradiol. Desoxyestrone(CXXXIV) was identified by preparation from estrone by Clemmensen reduction. The two products were found to be identical by mixture melting point determinations. When the Smith reduction hydrolysis was conducted with a more concentrated solution of estrone (30 mg./100 ml. solution), no desoxyestrone was obtained. 33% was recovered as estrone, the rest was non-ketonic and most of it was identified as α-estradiol. A fraction which could not be crystallized was assumed to be a mixture of the two epimers of estradiol.

The concentration of estrogens in urine resembles the concentration of estrone in the first experiment, and the formation of desoxyestrone can be postulated. As desoxyestrone (271-277) is not soluble in aqueous sodium hydroxide solution, it is lost on extraction of phenols, accounting for a considerable loss of activity. The biological activity of desoxyestrone has not yet been determined, but is assumed to be very low. The factor "5" accepted by Smith for the increase of biological activity on reduction of estrone, must be composed of varying percentages of factor "8 - 12" for reduction to α-estradiol, factor "0.3 - 0.08" for reduction to β-estradiol, an as yet unknown factor for reduction to desoxyestrone and a factor for the adsorption of estrogens on zinc.

3. Experimental Work.

Samples of urine (1 or 2 liter ; 6th to 8th month of gestation) were collected and treated as described in full detail on page 116 and 117, and referred to as treatment "A".

The ether insoluble interface material together with the filterpaper, on which it was collected, were then added to the extracted urine. The pH of the solution was adjusted to 0.3 by addition of concentrated hydrochloric acid, and 4% zinc dust was added. The mixture was refluxed for three hours in the fume cupboard (release of sulph-hydro compounds). The clear and colourless solution and some unreacted zinc was extracted with ether (3x600 ml.). The ether extracts were washed neutral with water, after all acids had been removed (9% sodium bicarbonate washings, 3x300 ml.) and taken to dryness. The residue was dissolved in benzene (200 ml.) and partitioned as described before (page 116,117). In a number of experiments, the "weak" phenols were separated into ketonic and non-ketonic fractions by the use of Girard's reagent "T", detailed on page 117. Hydrolysis with zinc, followed by extraction and partition, is generally described as treatment "B".

The fractions were then purified by chromatography. Adsorption columns were prepared with alumina (Harshaw,

Routine Fractionation of Urinary Estrogens.

Urine specimen

Treatment "A"

10' reflux with conc. HCl
(15 volumes %)
cooled quickly
extracted c. ether (4x)

ether and emulsion → filtered → ether → residue → washed c. ether

aqueous phase → treatment "B"

3^h reflux
c. 15% HCl & 4% Zn.
extracted as in treatment "A".

washed c. 9% $NaHCO_3$ (3x)
washed c. H_2O
taken to dryness
dissolved in C_6H_6

benzene

→ washed c. 10% Na_2CO_3 (3x) → carbonate solution → backwash c. C_6H_6

benzene

→ washed c. 1N NaOH (3x) → backwash c. C_6H_6

"Non-phenolic"

acidified c. HCl
extracted c. ether (3x)

"Weak Phenolic" → Girard "T" treatment → water soluble fraction / ether soluble fraction

"Strong Phenolic"

acidified c. HCl
16^h at 20^oC
extracted c. ether (3x)

"Estrone" → estrone

"Estradiol" → chromatographed on alumina
eluted with benzene, methanol
estimated quantitatively by
spectrophotometry. (λ_{max} 280mμ) → estradiol

"Estriol" → estriol

148.A

acetic acid washed, reactivated)(6 g.), making the height of the column 5 times its width. The columns were then washed with dry benzene, and the fraction to be chromatographed was dissolved in benzene (10 ml.) and adsorbed on the column. The weak phenolic materials were eluted with benzene containing increasing amounts of acetone (2, 4, 6, 10, 15, 20%) and finally with ether and methanol(100%). The strong phenols were eluted with benzene containing increasing amounts of methanol (2, 4, 6, 10, 15, 20, 100%).(Acetone was not chosen, as reactions between acetone and 1,2-glycols, as estriol, were expected to take place.) The eluates were taken to dryness, dissolved in 2 ml. ethanol and, after appropriate dilution, were analyzed spectrophotometrically. The solvent was then removed and crystallisation of all fractions was attempted.

1. Isolation and identification of estrogens from human pregnancy urine after treatment "A" and "B".

The results are condensed in chart 1. Estriol was identified by its melting point and by a mixture melting point determination with pure estriol. Its quantity was determined spectrophotometrically as well as gravimetrically. After treatment "A", crystals of estrone were obtained in small amounts, while the estradiol fraction consisted of an oil, which was not crystallized. The concentration of estrogens in these oily fractions were determined by their λ_{max} 280 mμ extinction, however their identification as estrogenic is not beyond doubt.

Table 1.

Treatment	mg. Estriol	mg. Estradiol	mg. Estrone
"A"	10.8	1.9 (?)	0.33
"B"	10.3	1.4 (?)	1.11 (?)

Fractions marked by a question mark could not be identified by crystallisation. They represent the quantity of material absorbing maximally at 280 mμ and eluted from the chromatogram in the fraction expected to contain the hormone.

In the "strong phenolic" fraction crystals of benzoic acid were isolated. They were purified by sublimation, and gave no depression in a mixture melting point with pure benzoic acid. The crystals sublimed at 116°C. Sodium carbonate washings will remove them from ether. The absorption spectrum of the crystals was determined. λ_{max} 272 and 280 mμ will interfere with the detection of estrogens.

2. Investigation of the discarded fractions for estrogens.

The ether extract, of a fresh batch of treated urine, was washed once with 10% sodium carbonate solution (éne tenth the volume of ether). The aqueous phase was acidified carefully and extracted with ether (3x30 ml.). The ether extracts were washed neutral, dried with sodium sulphate and taken to dryness. The residual oil was dissolved in benzene and chromatographed. The eluted oils were

analyzed by spectrophotometer. It was found that one sodium carbonate washing removed 10% of the total estriol present, besides removing acids and a substance possessing a strong absorption band at 256 mμ. An attempt was made to isolate the substance, since 6-keto-estrogens possess a similar band at 258 mμ, but all endeavours to crystallize the oil failed.

Subsequently acids were removed with 9% sodium bicarbonate solution, which does not remove any of the estriol, while still removing benzoic and other acids.

The ether solution, after having been washed with sodium carbonate, was extracted with N sodium hydroxide (3x200 ml.). The aqueous extracts were acidified, extracted with ether, washed and taken to dryness. They were then partitioned as described before.

The remaining ether, containing only "neutral" material, was washed neutral with water, taken to dryness and chromatographed. Two fractions were obtained which on spectrophotometric analysis showed the presence of phenolic material.

Consequently, ether extracts were washed with 9% sodium bicarbonate, washed neutral, taken to dryness, dissolved in benzene and partitioned, as it was seen that N sodium hydroxide will not remove all estrogens from ether. Similar results were obtained by Friedgood. From benzene all estrogens can be removed with N sodium hydroxide.

The results obtained with these minor modifications are given in table 2. As the"estriol fractions"were pure crystalline estriol, no difficulties were encountered in the calculation of their quantity by spectrophotometry. The"weak phenolic" eluates, however, were only partly crystalline and red oily materials were present.Identification of these fractions beyond doubt was impossible and the determination of the concentration of estrogens only succeeded after elimination of the "blank" absorption as described in part 2. (page 122).

Table 2.

	Estriol	Estradiol	Estrone
Treatment "A"	8.32mg.	1.94mg.(?)	2.01mg.(?)
" "B"	2.68 "	1.858" "	1.82 " "

3. Isolation and identification of estrogens in human pregnancy urine.

Pregnancy urine (4 l.,8th month) was autoclaved (120°C,15 pounds) in the presence of 15 volumes % concentrated hydrochloric acid for 10 minutes, cooled and extracted with ether (4x600 ml.)(Treatment "C");The aqueous phase was autoclaved for 3 hours in the presence of zinc dust (2%), cooled and extracted with ether.Both ether extractions were combined and purified,partitioned and chromatographed as was previously described. Crystallisation of all fractions revealing a 280 mμ absorption maximum was attempted. A search for desoxyestrone and other phenolic compounds in the "neutral fraction"was also made.

Table 3.

Non-ketonic weak phenols

1.Bz. 100%	oil	---		
2. " 2% acetone	"	---		
3. " 4" "	"	1.856 mg		??
4. "10" "	"	---		
5. "15" "	"	3.79 "		??
6. "20" "	Crystals	3.465 "	170-176°C	∀-estradiol
7.Ether 100%	"	1.38 "	214-226°C	ß-estradiol
8.CH_3OH "	"	15.56 "	279-281°C	estriol

Ketonic weak phenols

1.Bz. 100%	oil	---		
2. " 2%acetone	"	---		
3. " 4" "	"	---		
4. " 10" "	crystals	250-254°C	0.9105 mg.	estrone
5. " 15" "	"	"	0.9222 "	"
6. " 20" "	oil	---		
7.Ether 100%	"	---		
8.CH_3OH "	crystals	274-278°C	7.63 "	estriol

Strong phenols

1.Bz. 100%	Oil	---		
2. " 2% methanol	"	---		
3. " 4" "	"	20.1 mg.		??
4. " 10" "	"	9.1 "		??
5. " 15" "	"	---		
6. " 20" "	Crystals	19.0 "	270-274°C	estriol
7. 100" "	"			
8. " " "	----			

In the neutral fraction the absorption maximum at 280 mμ could not be detected, but its absence could also not be established on account of large amounts of interfering substances.

4. Comparison of consecutive treatment of pregnancy urine with parallel treatment of aliquots of urine (Smith's method).

One aliquot (one liter)(7th month of gestation) was given treatment "A" and "B", while the other was only given the latter treatment. It was surprising to find that the yield of estrogens obtained by the two procedures was not the same. Treatment "B" alone gave much lower yields of estrogens than the combined 10-minute and 3-hour hydrolytic treatments. This discovery was confirmed by weighing the crystals of estriol, besides spectrophotometric analysis. Estrone was not discovered in any of the fractions.

Table 4.

	Treatment	Estriol	Estradiol	Estrone
1.	"A"	11.7 mg	1.7 mg.	--
	"B"	13.6 "	1.91 "	--
2.	"B"	8.2 "	0.71 "	--

5. The action of hydrogen on urinary estrogens at 20°C at pH 0.3.

Two liters of 7th month pregnancy urine were extracted with ether to remove free estrogens. To the urine, zinc dust (4%) and concentrated hydrochloric acid (15 volumes %) were added, and the mixture, liberating large quantities of hydrogen, was allowed to stand at room temperature for 16 hours. The urine was then extracted and the extract was fractionated in the

usual manner. No estrogenic material was obtained from any of the fractions and it was concluded, that reduction alone does not suffice to reactivate the hypothetical estrogen oxidation product. One half of the urine sample was then given treatment "A" and "B", while the other half received only "B" and the same observation was made again as in section 4.

Table 5.

	Treatment	estriol	estradiol	estrone
1.	"A"	7.97mg.	0.835mg.	0.64mg.
	"B"	4.32 "	0.123 "	0.14 "
2.	"B"	5.4 "	0.45 "	0.17 "

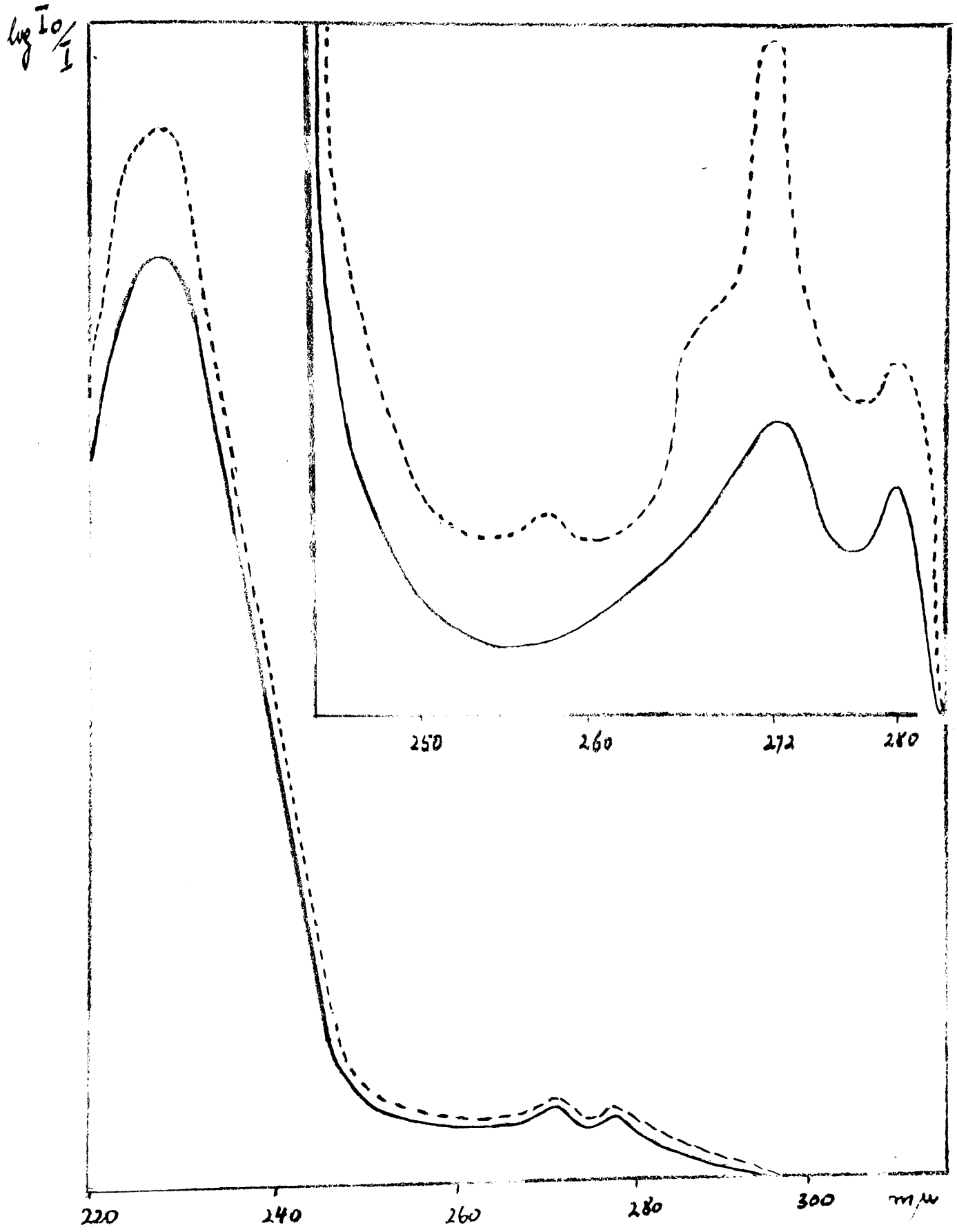
6. Examination of the strongly adsorbed fraction of the chromatograms in section 5.

After the estrogens had been eluted from their chromatograms, the columns were washed with N sodium hydroxide solution (25 ml.). The eluate was acidified, extracted with ether (3x15 ml.), and the ether extracts were washed neutral with water, dried with sodium sulphate, condensed to a smaller volume (10 ml.) and methylated with diazomethane. Excess diazomethane and the solvent were removed with care, and crystals were not obtained on chromatographic separation. Some of the oily fractions showed a 280 mμ absorption maximum. On examination of a new N sodium hydroxide solution eluate, crystals were obtained in small amounts and their identity was investigated.

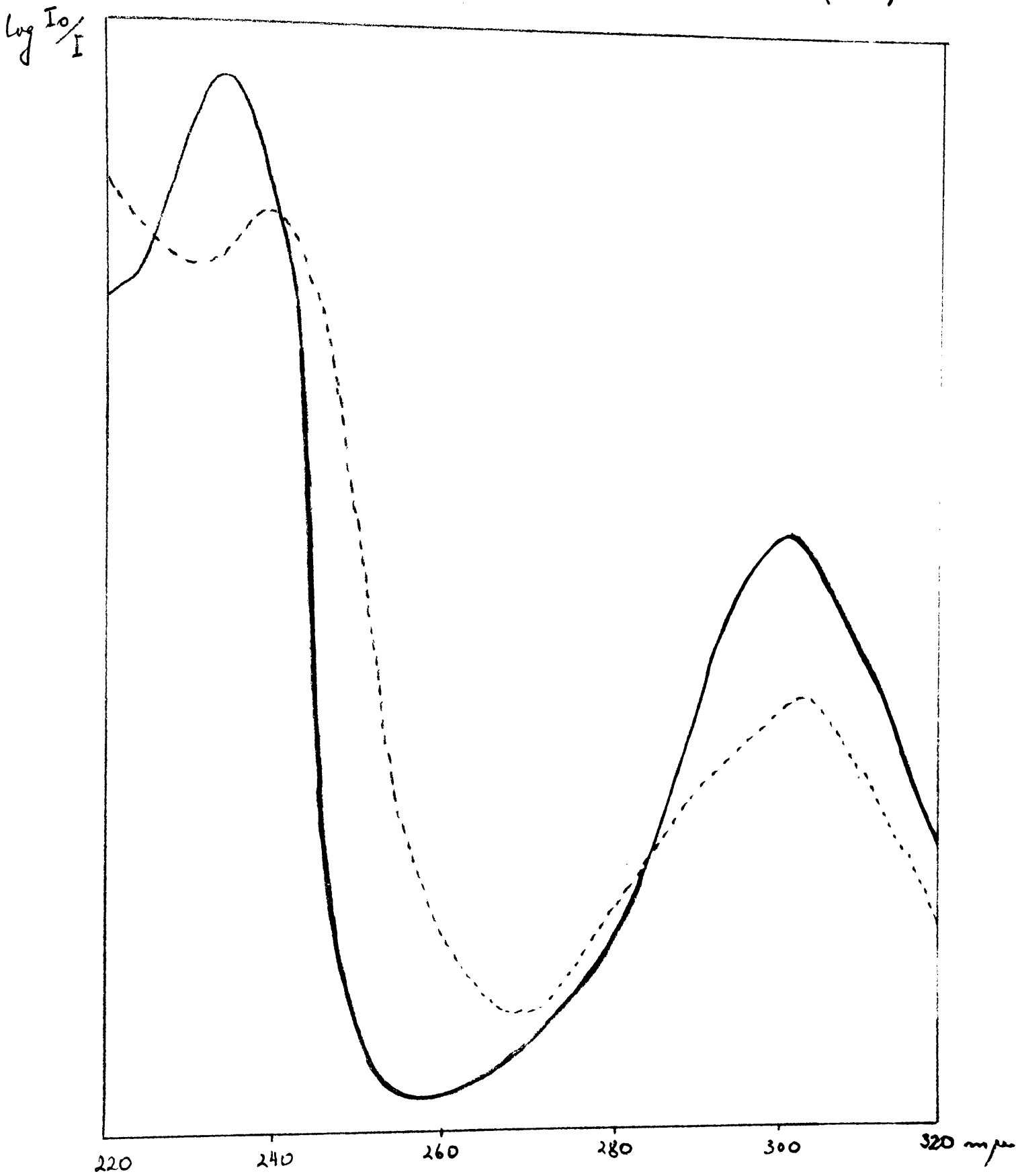
The crystalline material was washed with petrol ether, in which part of the crystals dissolved. On evaporation of the solvent, colourless crystals were obtained. m.p. 104-110°C. They gave a purple colour with ferric chloride, and their absorption spectrum is very similar to that of benzoic acid. λ_{max} 226, 272, 280 mμ, the extinction coefficient of the latter two being only one tenth of the former. Like benzoic acid it is volatile with steam and sublimes at 100°C. It differs, however, from benzoic acid in its reaction with ferric chloride, its melting point, its elution from activated alumina and its empirical formula. A carbon and hydrogen determination, made by Mrs. Jewitt of Ayerst, McKenna and Harrison Ltd., gave the following values: C: 59.06% H: 4.37%
59.33% 4.14%,
suggesting a formula of $C_7H_6O_3$, meta or para hydroxy-benzoic acid presumably. No further investigations could be made on the compound, since it could not be isolated from other samples of pregnancy urine.

The petrol ether insoluble crystals also gave a deep purple colour with ferric chloride. They melted at 162-167°C. For further purification, the crystals were chromatographed on a column, consisting of magnesium silicate and Celite (2:1 by weight). The two adsorbants were mixed with carbon tetrachloride and poured through a long tube to the bottom of the column, consisting of a sintered glass plate. Care was taken to keep the column moist.

The absorption spectrum of Benzoic Acid (----)
and of compound $C_7H_6O_3$ from urine (——).



The absorption spectrum of Salicylic acid(——)
and of the unidentified compound of urine(---)



The crystals were dissolved in ether (1 ml.), and the solution was diluted with 20 ml. carbon tetrachloride and adsorbed on the column. Crystals, m.p.183-184°C, were eluted with ether, which give a purple colour with ferric chloride and two absorption bands, at 236 and 302 mμ on spectrophotometric analysis. The absorption spectrum is similar to that of salicylic acid, which also shows the same colour reaction with ferric chloride. The melting point of salicylic acid, however, is much lower than that observed here. Since these crystals could not be isolated again, they were not considered to be estrogen breakdown products. The absorption spectra of the compounds are shown on page 157 and 158.

7. Butanol fractionation of urine after treatment "A".

After 10-minute hydrolytic treatment and extraction, aliquots of pregnancy urine were extracted with n-butanol (3x300 ml.). Most of the n-butanol was removed on the steam bath under suction, 500 ml. of water was added, and the conjugates present were hydrolyzed by treatment "B". The aqueous phases were given the same treatment. Details and results are given in table 6. It was observed, that practically all additional estrogenic material was obtained from the butanol.

Table 6.

Sample	Treatment	Strong phenols	Weak phenols
1.	"A"	31.6 mg	1.6 mg.
butanol	"B"	5.97 "	0.74 "
aqueous	"B"	0.48 "	0.05 "
2.	"A" & Zn	24.6 mg.	0.61 mg.
butanol	"B"	10.5 "	0.90 "
aqueous	"B"	1.0 "	0.27 "

8.Modifications of hydrolysis,fractionation and chromatography.

Pregnancy urine was obtained from a new donor, and difficulties were encountered on spectrophotometric analysis of fractions obtained from urine after treatment"A". Strong absorption maxima at 290 and 310 mμ interfered with the detection and calculation of the amounts of estrogens present. As this interfering absorption spectrum was not observed after treatment "B", the ether extracts after "A" were treated with zinc and hydrochloric acid,to destroy the interfering substance.

Instead of refluxing the solutions in a glass-col heating element, treatment "A" and "B", the solutions were given otherwise identical treatment in the autoclave at 120°C and 15 pounds pressure,in the absence of air.Treatment"C"and"D". Separation of the weak phenolic fraction into ketonic and non-ketonic phenols was eliminated, as more or less complete reduction was taking place in all extracts.The shorter chromatographic procedure of Stimmel was also introduced,using benzene and 1, 2, 4, 6, 10% methanol and 100% methanol as eluants. With all these modifications similar results were obtained as before. Table 7.

Table 7.

	Treatment	Strong phenols	weak phenols
1.	"A"	7.37mg.	3.22mg.
	"B"	12.9 "	1.28 "
2.	"A" & Zn	4.5 mg	2.14 "
	"B"	17.6 "	1.01 "

9. Fractionation of urine with n-butanol.

One of four aliquots of pregnancy urine(1 liter,8th month) was acidified to pH 3 and extracted thoroughly with n-butanol.The n-butanol extracts(1200 ml.) were extracted with N sodium hydroxide solution (3x300 ml.),washed neutral and condensed on the steam bath under vacuum.The residue was diluted with 500 ml. of water. The aqueous alkaline extracts were neutralized.All three solutions were given treatment "C", followed by "D". The remaining three aliquots of urine were given hydrolytic treatment in the autoclave or in air, with or without zinc as described in table 8.

Table 8.

Sample	Treatment	strong phenols	weak phenols
1.urine	"C"	1.45 mg	0.27 mg
butanol	"C"	0.59 "	1.0 "
NaOH	"C"	6.95 "	0.04 "
1.urine	"D"	0.33 "	0.06 "
butanol	"D"	---	0.00 "
NaOH	"D"	0.59 "	0.05 "
2.	"C" & Zn	2.48 "	0.33 "
	"C" & Zn	2.346 "	0.72 "
3.	"C"	5.981 "	0.38 "
	"C" & Zn	3.435 "	0.96 "
4.	"A"	6.773 "	0.76 "
	"D"	3.686 "	0.62 "

It was seen that sodium estriol glucuronate could be hydrolyzed in 10 minutes, if it was heated in acidified

distilled water, while on hydrolysis in urine only 66% were obtained under identical conditions. The weak phenols, which remaind in the n-butanol extract, were also completely hydrolyzed in 10 minutes. Low yields of estrogens were again obtained, when zinc was added during the first hydrolysis.

10. The second hydrolysis with and without zinc.

After 10-minute hydrolyses, the remaining urinary conjugates were hydrolyzed with and without zinc for 3 hours in the autoclave. The latter case was designated as treatment "E". The sample, receiving treatment "E", was given an additional hydrolytic treatment with zinc during one hour in the autoclave, to see whether additional amounts of estrogens would be freed, requiring the presence of hydrogen. From the results in table 9 it was concluded, that zinc was not necessary for the recovery of further quantities of estrogens. It was postulated that zinc increases the yield of estrogens, however, by production of a hydrogen atmosphere, thus preventing their oxidative destruction.

Table 9.

	Treatment	Strong phenols	Weak phenols
1.	"C"	4.0 mg.	0.61 mg.
	"E"	3.13 "	0.62 "
	"D" (1 hour)	0.74 "	0.22 "
2.	"C"	4.0 "	0.61 "
	"D" (4 hours)	5.12 "	1.15 "
3.	"C" & Zn	3.13 "	1.33 "
	"D"	4.1 "	0.71 "

11. Hydrolysis of the conjugates with protection from oxidative destruction.

Five aliqots of pregnancy urine (1 liter, 8th month) were given hydrolytic treatments. For protection from oxidative destruction, the air was displaced by steam in the autoclave, a stream of nitrogen was used or a hydrogen atmosphere was produced by zinc and hydrochloric acid. One aliquot served as control and was refluxed in the presence of air without zinc. All samples received otherwise exactly the same treatment. As was expected, the lowest yields were obtained in the sample serving as control, while approximately equal quantities of estrogens were recovered from the other samples. When zinc was added to the first hydrolytic treatment, low yields were obtained, however, higher yields were obtained on second hydrolysis in these cases, making the total quantity of estrogens recovered the same, as in other samples.

Table 10.

	Treatment	Strong phenols	Weak phenols
1.	"A" & Zn	6.45 mg	0.60 mg.
	"B"	4.25 "	0.69 "
	"D"	1.92 "	0.27 "
2.	"C" & Zn	3.54 "	0.75 "
	"D"	8.72 "	1.16 "
3.	"A"	5.31 "	0.88 "
	"A" (3 hours)	3.70 "	0.34 "
	"D"	2.28 "	0.22 "
4.	"A" under N_2	5.28 "	0.56 "
	"A" " " (3 hours)	4.03 "	1.07 "
	"B" " "	1.08 "	0.25 "
5.	"B"	2.87 "	0.85 "
	"B" (3 hours)	4.12 "	0.88 "
	"D"	2.68 "	0.41 "

From table 10 it was deduced, that only 40 to 50 % of the estrogens can be liberated in 10-minute hydrolysis "A" or "C". A further 40% could be freed by treatment "B" or "D", while 10 to 20% had not been hydrolyzed even after that treatment and required an additional hydrolytic treatment. The most important observation was, that other means of protecting the estrogens from oxidative destructions were just as effective as the hydrogen atmosphere produced by the action of zinc on hydrochloric acid.

12. The length of hydrolysis and the yield of estrogens.

Aliquots of pregnancy urine (1 liter, 8th month) were autoclaved with and without zinc, two further aliquots were autoclaved after butanol and sodium hydroxide fractionation and on two further aliquots the rate of hydrolysis was examined. Six consecutive 10-minute treatments were made, and the quantity of estrogens liberated after each hydrolysis was found to decrease quickly, whether zinc was present or not. The total yield after 6 periods of hydrolysis only amounted to 40% of the amounts of estrogens liberated after one 3-hour hydrolysis. This finding confirmed Marrian's observation (230), that estrogens are easily destroyed, when hydrolysis is carried out in the presence of air. It appears probable, that the ether, present in solution in the aqueous phase after extraction, would form ether peroxides, which could account for the observed destruction.

Table 11.

Sample	Treatment		estriol	weak phenols
1.urine	"C"	(3 hours)	1.61mg.	0.15 mg.
butanol	"C"	"	0.61 "	1.42 "
NaOH	"C"	"	8.67 "	----
2.urine	"D"		2.03 "	0.24 "
butanol	"D"		0.81 "	0.85 "
NaOH	"D"		10.36 "	----
3.urine	"C"	(3 hours)	10.82 "	2.03 "
4.urine	"D"		14.23 "	2.67 "
5.urine 1st	"C"		1.95 "	0.773 "
2nd	"C"		1.32 "	0.227 "
3rd	"C"		0.57 "	0.079 "
4th	"C"		0.43 "	0.046 "
5th	"C"		0.97 "	0.076 "
6th	"C"		0.55 "	0.066 "
6.urine 1st	"C"	& Zn	1.82 "	0.622 "
2nd	"C"	"	0.80 "	0.213 "
3rd	"C"	"	0.38 "	0.103 "
4th	"C"	"	0.11 "	0.038 "
5th	"C"	"	0.11 "	0.027 "
6th	"C"	"	0.07 "	0.031 "

13.Hydrolysis of ethereal sulphates with barium chloride.

Barium chloride (24.4 g.) was dissolved in water and added to one aliquot of pregnancy urine(1 liter) at pH 5. A copious precipitate formed. The mixture was autoclaved for ten minutes, extracted and again autoclaved (3 hours).Two aliquots served as controls. They were subjected to 3 successive 10-minute acid treatments in the autoclave with or without zinc. On barium chloride hydrolysis no estrogenic material was liberated. The aqueous phase was subjected to treatment"C" and the expected estrogens were obtained.The investigation was ended.

Table 12.

Sample	Treatment	estriol	weak phenols
1.	1st "C"	4.14mg.	0.93mg.
	2nd "C"	2.64 "	0.35 "
	3rd "C"	1.06 "	0.07 "
2.	1st "C" & Zn	5.76 "	1.15 "
	2nd "C" "	2.96 "	0.50 "
	3rd "C" "	1.09 "	0.06 "
3.	1st $BaCl_2$	---	---
	2nd "	---	---
	"C"	6.35 "	0.48 "

14. Purification of calcium estriol glucuronate.

Three g. of material, which analyzed spectrophotometrically 11% estriol, was shaken up with N/3 sodium hydroxide solution(6x25 ml.) and filtered.The residue was found to be free of λ_{max} 280 mμ absorbing material and was discarded. The alkaline solution was acidified to pH 2.5-2.7 and extracted with n-butanol(4x60 ml.).The absorption spectrum of the aqueous solution did not show a maximum at 280 mμ after 4 extractions. The butanol extracts revealed the presence of 330 mg. estriol as the glucuronate.

The butanol solution was extracted with N/3 sodium hydroxide solution (7x75 ml.),until free of "estriol".The alkaline extracts were acidified to pH 2.7 and washed with ether (3x100 ml.), to remove free estriol,(back-extracted once with water) and extracted with n-butanol,(4x150 ml.).

When the ether extract was washed, the ether was distilled,leaving a large volume of butanol behind.Due to the

presence of the conjugate in n-butanol, the separation of free estriol had to be repeated. The butanol was removed on the steam bath under vacuum; the residue was dissolved in N/3 sodium hydroxide solution, acidified to pH 2.7 and extracted with ether(3x50 ml.) The presence of small amounts of estriol in the ether extract was discovered.The aqueous phase was extracted with butanol, which was combined with the main solution of n-butanol.The butanol extract was washed neutral with dilute ammonia solution(3x100 ml.)(back-extracted with butanol) and taken to dryness on the steam bath under vacuum. The residue was dissolved in methanol (25 ml.), condensed to a small volume and allowed to stand in the refrigerator. Sodium chloride settled out and was removed by filtration. The brown solution was condensed to 4 ml. and made alkaline to pH 7.8 by addition of a few drops of N sodium hydroxide solution. On cooling crystals formed,m.p.245°C with decomposition, which were filtered off.On spectrophotometric analysis they were found to be 82% pure sodium estriol glucuronate.

From the mother liquors more crystals of the glucuronate were obtained. The remaining mother liquors were taken to dryness. 250 mg. estriol were obtained as crystalline conjugate, the remaining 80 mg. were detected in the mother liquors. Purification by means of the calcium salt of estriol glucuronic acid of the material in the mother liquors failed.

15. Hydrolysis of sodium estriol glucuronate at pH 1.

The glucuronate (2.4 mg.)(CXXXII) was dissolved in hot water (5 ml.) and diluted to 100 ml. The solution was analyzed spectrophotometrically and found to contain 1.003 mg. estriol. The solution was acidified to pH 1 and autoclaved for 10 minutes (120°C, 15 pounds). On extraction with ether, no estriol was detected in the extract. It was all still in the water soluble form. The solution was autoclaved at pH 1 with 4% zinc. After 3-hour hydrolysis 0.8259 mg. estriol was extracted with ether. The pH of the solution was readjusted from 3.5 to 1 and hydrolysis was continued for an hour, liberating 9% estriol, 0.0941 mg.

Table 13.

10	minutes,	autoclave,	pH 1,	00%	estriol	hydrolyzed
3	hours	"	& Zn "	82.6%	"	"
1	hour	"	" "	9.2%	"	"
			total	91.8%		

16. The rate of hydrolysis and destruction at pH 1.

Estriol glucuronate (13.2 mg.) in warm water was diluted with 400 ml. water and the concentration was determined accurately by spectrophotometry: Four 100 ml. aliquots containing 2.9mg. estriol glucuronate. All solutions were autoclaved at pH 1, two with and two without zinc. One of each pair was extracted after every 10-minute hydrolysis, while the other was analyzed for total estrogens. For spectrophotometric analysis, the solutions containing zinc had to be filtered. The zinc was, however, returned to the solution.

Table 14.

1. autoclave pH 1,	1st 15'		24%	estriol liberated
	2nd "		17%	" "
	3rd "		11%	" "
	4th "		11%	" "
	60'		36%	" "
		total	99%	" "
2. autoclave pH 1,	1st 15'	& 4%Zn	48%	" "
	2nd "		8%	" "
	3rd "		11%	" "
	4th "		15%	" "
	60'		17%	" "
		total	99%	" "
3. autoclave pH 1,	2 hours		100%	detected in solution
			90%	extracted with ether
4. autoclave pH 1,	2 hours & Zn		100%	detected in solution
			92%	extracted with ether

From the above results it could be concluded that Marrian's method of hydrolysis gives complete hydrolysis without destruction. The observation that only 90% estriol was extracted after 2-hour hydrolysis with ether(3x50 ml.) was explained later on by the observation, that only 90% of estriol present is extracted with ether in 3 extractions. For complete extraction 4 or 5 ether extracts seem necessary.

17. Hydrolysis in the presence of air at pH 1.

The conjugate(16.4 mg.) was dissolved in 303 ml. water and the concentration was determined spectrophotometrically. Three 100 ml. aliquots were hydrolyzed under reflux or in the autoclave as described in table 15. In the autoclave or under reflux in the presence of zinc 75% of the estriol was recovered.

When the solution was not protected from atmospheric oxygen, only 42.5% estriol was recovered. The aqueous solutions were adjusted to pH 1 and hydrolyzed for one hour. No further yields of estriol could be extracted. The solution in which 58% of estriol had not been recovered, yielded on second hydrolysis a yellow oil, which did not possess the 280 mμ maximum. From the other 2 solutions, ether extracts contained no material.

Attempts at the identification of the yellow oil by spectrophotometric analysis were made. The absorption spectrum revealed 3 bands, at 248, 254 and 260 mμ. To establish this absorption spectrum as belonging to the hypothetical product of oxidative destruction of estrogens, control experiments were made. These experiments established the fact, that the spectrum was due to the ether (one of its contamination products) and not due to the estriol oxidation product. The spectrum is recorded on page 171.

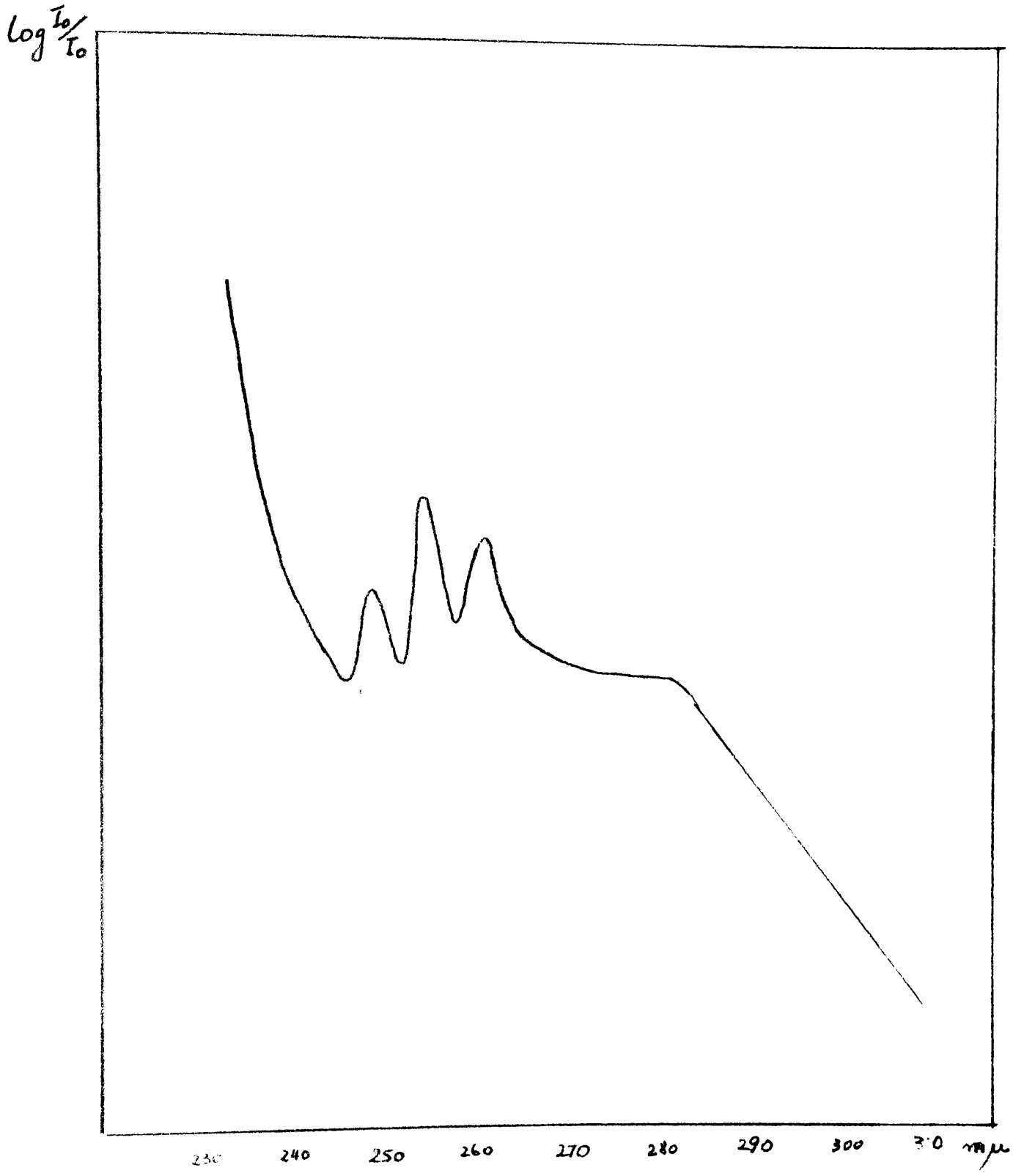
Table 15.

1.	reflux, 2 hours,	pH 1,	42.55% estriol	recovered	in	ether
2.	autoclave " & Zn	" "	74.5 %	"	"	"
3.	reflux, " & Zn	" "	75.44%	"	"	"
1.	reflux 1 hour	" "	-------- oil	"	"	"
2.	" "	" "	--------- ----			
3.	" "	" "	--------- ---			

18. Hydrolysis of estriol glucuronate by Smith's method.

Sodium estriol glucuronate (13.2 mg.) was dissolved in 300 ml. water. Every 100 ml. aliquot was found spectrophotometrically to contain 1.858 mg. estriol. The solutions were

Absorption spectrum of impurity in the ether.



heated to boiling by a glass-col heating element, before 10 ml. concentrated hydrochloric acid was added through the condenser. After 10 minutes, the solutions were rapidly cooled and extracted with ether(3x40ml.).52% of the estriol present was recovered in the ether extracts. The remaining aqueous solutions were combined and allowed to stand at room temperature for 16 hours.From the solution 5% of the total estriol was extracted with ether.

The aqueous phase was then extracted with n-butanol, to see, whether the unaccounted for 42% estriol had been destroyed or were still in conjugated water soluble form. The butanol extracts were washed neutral with water and taken to dryness on the steam bath under vacuum. 39% estriol was recovered from the butanol and it was concluded, that no destruction had occurred, but that hydrolysis during 10 minutes had been very incomplete.

Table 16.

1/3	10' reflux	15% HCl	53.6% estriol	liberated
"	" "	"	51.8% "	"
"	" "	"	51.1% "	"
1	16^{h} at 20^{o}C	"	4.6% "	"
1	n-butanol extract		39.0% "	conjugated
		total	96.6% "	recovered.

19. Comparison of the efficiency of hydrolytic methods described in the literature.

Eleven methods of hydrolysis of the estrogen conjugates in urine were tested. 25 ml. samples containing 150 µg. estriol as its glucuronate in distilled water were treated as described in table 17.

Table 17.

Investigator	Ref.	Conditions	Recovery of estriol
Marrian	156	pH 1 2^h autoclave	99.6%
Callow	246	4% HCl 1^h "	94 %
Venning	235	pH 3 & 0.6%HCl 2^h "	89.5%
Pincus	248	15%HCl 7' reflux	81 %
Cherry	244	5% HCl 1^h "	74.8%
Smith	241	15%HCl 15' "	70 %
Koch	233	10%HCl 15' "	68.3%
McCullough	242	5%H_2SO_4 15' "	47.7%
Smith(Zinc)	234	15%HCl 10' & 4% Zn "	43.6%
Laqueur	240	4% HCl 15' & C_6H_6 "	25 %
Palmer	245	14%HCl 90' autoclave	0 %

Marrian's method was found to give quantitative recovery, while hydrolyses of short duration with high concentration of acid gave 70 to 80% yields.Hydrolysis in the autoclave was also observed to give better results.

20. Variations in Smith's method of hydrolysis.

Seven solutions of sodium estriol glucuronate (0.8531 mg"estriol" in 100 ml. water) were hydrolyzed in the presence of enough concentrated hydrochloric acid, to produce the same pH as on addition of 15 volumes% acid to urine. (10 volumes % concentrated hydrochloric acid). The variations are described in table 18. After each period of hydrolysis, the solution was extracted with peroxide free ether, the ether was washed neutral, taken off at room temperature, and the residue was analyzed spectrophotometrically.

Table 18.

1.	10% HCl		10'	reflux	63.2%	estriol	recovered
	"	4%Zn	3h	"	28.5"	"	"
				Total	91.7%	"	"
2.	"	4%Zn	3h10'	"	52.4%	"	"
3.	"	4%Zn	10'	"	30.3%	"	"
		"	10'	"	16.0"	"	"
				Total	46.3%	"	"
4.	"		10'	"	69.2%	"	"
			10'	"	--	"	"
			10'	"	--	"	"
5.	"		20'	"	68.0%	"	"
			10'	"	19.0%	"	"
				Total	87.0%	"	"
6.	"		10'	"	64.5%	"	"
		4%Zn	10'	"	32.0"	"	"
				Total	96.5%	"	"
7.	"	4%Zn	20'	"	40.0%	"	"
		--	10'	"	--	"	"

From these results a number of important deductions were made. 65% of the conjugate was hydrolyzed during 10 minutes, while the rest was still present in the conjugated form. If the solution was heated for a longer period without extraction no destruction of the liberated estriol occurred. After extraction with ether, further hydrolytic treatment only produced oils, which did not show the characteristic spectrum of the estrogens. This destruction could be prevented by the addition of zinc in the second hydrolysis. The formation of ether peroxides can be prevented by the hydrogen atmosphere produced. On the other hand, zinc readily adsorbes a part of the estriol making its extraction difficult and also raises the pH of the solution, so that further acidification is necessary for effective hydrolysis.

Estriol (0.084 mg.) was heated in strongly acidified water containing as much ether as would dissolve in it, in order to clarify the conditions of estrogen destruction. On extraction no maximum could be observed at 280 mμ, suggesting complete destruction of the estriol on 10-minutes "hydrolytic treatment" in the presence of ether.

21. Hydrolysis of the conjugate in the presence of solvents.

Seven solutions (100 ml. water) containing 1.032 mg. estriol as its glucuronate were prepared. The fat solvent was added to the solution in a separatory funnel, shaken vigorously and allowed to separate. The aqueous layer was then hydrolyzed.

After extraction with peroxide free ether, the solutions were autoclaved for 10 minutes in the presence of 4% zinc and again extracted with ether. The concentration of estriol in the extracts was determined spectrophotometrically.

Table 19.

1. 10%HCl 10' reflux		2. 10%HCl & 4% Zn 10' autoclave		
-------	49%	40%	Total 89%	recovered
ether	33%	40%	" 73%	"
ether, Zn	33%	19%	" 52%	"
----- Zn	33%	28%	" 61%	"
ether, N_2	35%	44%	" 79%	"
$CHCl_3$	36%	46%	" 82%	"
C_6H_6	40%	45%	" 85%	"

The highest yield of estriol was obtained in the absence of ether, although under these conditions a considerable amount of estriol was still recovered in the presence of ether. The secondary effects of zinc were again noted. The effect of other fat solvents was not very clear cut.

22. The extraction of estriol.

100 ml. aliquots containing 0.67 mg estriol in water were heated in the presence of 10 volumes % hydrochloric acid with and without zinc (4%) and extracted. Without zinc, 94% of the estriol was recovered, but in the presence of zinc only

53 to 59% was obtained. As a 100% recovery was not achieved the question of destruction or incomplete extraction was investigated. Estriol (1.32 mg.) was suspended in 100 ml. acidified water and extracted with 35 ml. of ether. The extracts were not combined, but were washed neutral (1x10 ml.), taken to dryness and analyzed spectrophotometrically. Estriol was recovered quantitatively. 1st: 42%, 2nd: 30%, 3rd: 18% and 4th: 9%. However, it proved that 3 extractions with ether will only remove 90% of the estriol present, accounting for a number of low yields.

23. The recovery of estriol in the presence of zinc.

Estriol (0.106 mg.) was dissolved in a few drops of methanol and diluted to 10 ml. with water. The concentration of estriol was determined spectrophotometrically. 4% zinc was added and the suspension was shaken. As the zinc did not settle out, part of the solution was filtered and analyzed by spectrophotometer. The original amount of estriol was still in solution. Zinc was again added and the solution was acidified with hydrochloric acid. The suspended zinc precipitated immediately, and the supernatant fluid revealed the presence of 82 µg. estriol by its extinction. 20% of the estriol was not recovered.

24. Recovery of estriol after hydrolysis of the conjugate in the presence or absence of zinc.

Solutions containing 0.604 mg. estriol as the glucuronate in 90 ml. of water were prepared and analyzed.

One aliquot was hydrolyzed in the absence of zinc, while the other three were hydrolyzed in the presence of varying amounts of zinc, paying attention to the complete solution of all zinc present in one solution. The solutions were extracted and the aqueous phases were refluxed for 50 minutes in the presence of zinc and acid, making sure, that in the same solution all zinc should have disappeared before extraction. (solution 3)

Table 20.

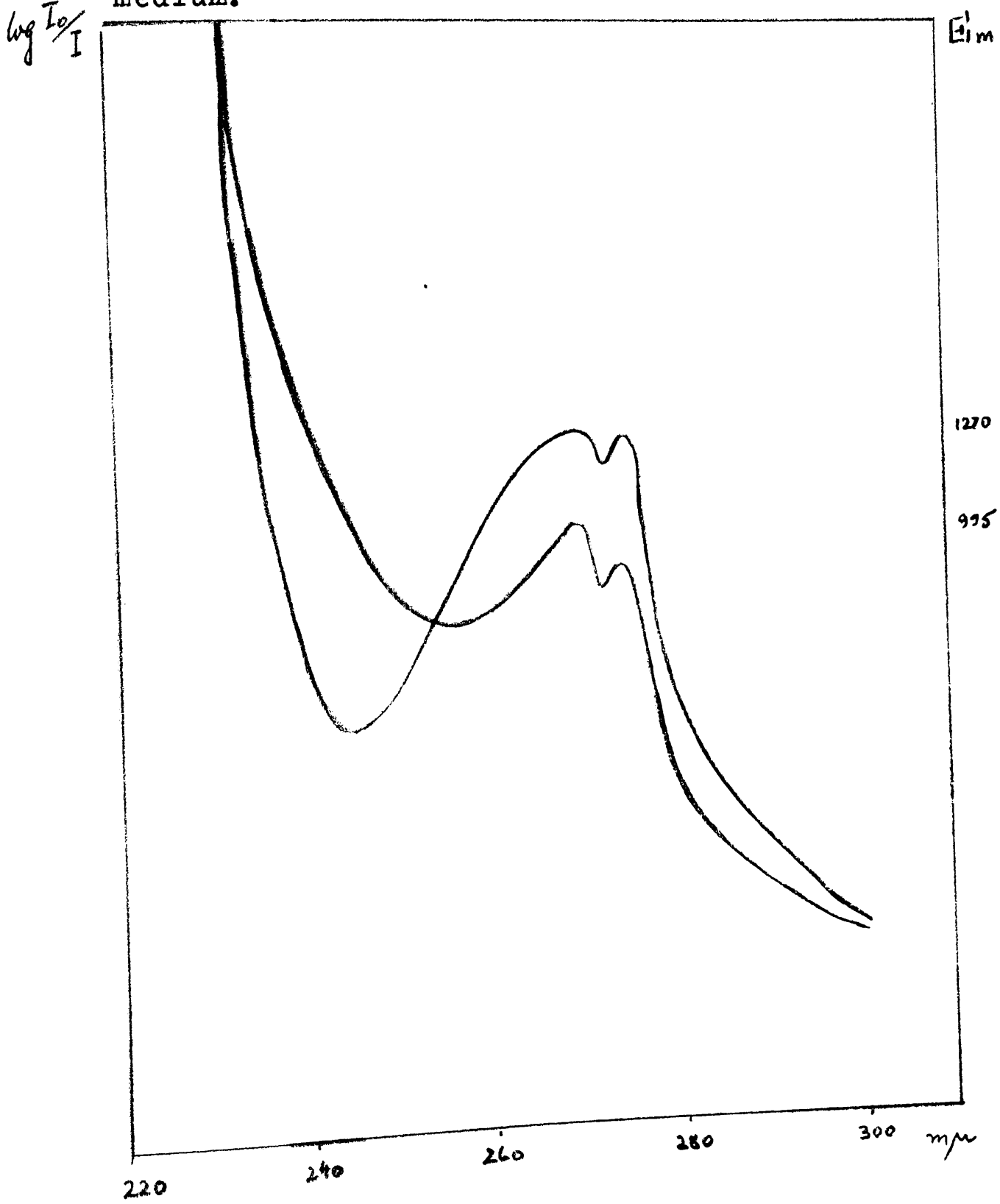
1.	15%HCl,	4%	Zn,	10'	reflux,	Zn left	55.4%	2nd	10',	Total	83%.
2.	10%	"	1%	"	"	"	"	"	33.1%	"	"	"	75%
3.	15%	"	1%	"	15'	"	no Zn	"	77.0%	"	15'	"	100%
4.	10%	"	-------	"	"	"	"	71.5%	"	10'	"	90%

From these observations it can be concluded that adsorbed estriol can again be liberated unchanged on solution of the zinc, giving quantitative recoveries of the liberated estriol. On drastic measures of solution of excess zinc(prolongued heating on the steam bath or addition of concentrated hydrochloric acid to the metal at room temperature), all the estriol is destroyed.

25. The absorption spectrum of sodium estrone sulphate.

Estrone sulphate (3 mg.) was dissolved in water(2 ml.). 0.2 ml. of the solution was diluted with 2 ml. of water and the absorption spectrum was determined in neutral, acid and alkaline

Absorption spectrum of Estrone sulphate in alkaline(λ_{min} 254mμ) and acid(λ_{min} 244mμ) medium.



solution. Absorption maxima were observed at 268 and 274 mμ. On shifting from an acid or neutral to an alkaline medium, the position of the maxima did not change, but the minimum was shifted from 244 to 254 mμ. The absorption spectrum is given on page 179.

26. Hydrolysis of sodium estrone sulphate.

Estrone sulphate (2.7 mg.) in 20 ml. water was allowed to stand at room temperature for 45 minutes. No hydrolysis occurred under those conditions.

The solution was acidified with 10 volumes % concentrated hydrochloric acid and refluxed for 10 minutes. On extraction with ether, 1.954 mg. crystalline estrone was recovered, representing a quantitative recovery of estrone. (2.7 mg. sodium estrone sulphate contain 1.956 mg. estrone).

27. Hydrolysis of estrone sulphate under different conditions.

Six aliquots (525 μg. estrone sulphate in 50 ml. water) were autoclaved for 10 or 60 minutes at a pH 1 or 0.3 and with or without zinc (4%). At pH 1 only 74% estrone was recovered, whether hydrolysis was carried out over 10 or 60 minutes, while on hydrolysis with 10 volumes % concentrated hydrochloric acid quantitative yields were obtained on 10-minutes treatment. If any destruction occurred, it could only amount to 5% in one hour. On hydrolysis in the presence of zinc, no estrogenic material could be recovered. An oily residue was obtained, which

did not possess a 280 mμ absorption band. On purification no estrogenic absorption spectrum could be detected.

Table 21.

1.	10'	autoclave	pH 1	387.6 μg,	74%,	estrone recovered
2.	1h	"	" "	387.0 μg,	74%	" "
3.	10'	"	10%HCl	522.6 μg,	100%	" "
4.	1h	"	"	493.4 μg,	95%	" "
5.	10'	"	" Zn	-----	---	" "
6.	1h	"	" "	-----	---	" "

28. Separation of estrone sulphate from estrone.

Crystalline estrone sulphate had turned pink and become partially hydrolyzed, while standing in a desiccator. The crystals were dissolved in the minimum amount of methanol (20 ml.), and 20 volumes of ether were added. Crystals of estrone sulphate separated and were removed from the solution by centrifugation. From the supernatant fluid 90% of the material was recovered as estrone on evaporation of the solvent. The crystals of estrone sulphate were washed with ether, dried in a desiccator over phosphorus pentoxide and stored in the dark.

29. The effect of zinc on estrone.

A solution of estrone in ethanol(5 ml.) was prepared and the concentration was determined spectrophotometrically.

1.84 mg. estrone was present. 4% zinc dust was added, the solution was shaken and filtered and analyzed. Only 1.605 mg. of estrone were detected. The zinc was returned to the solution and 8 drops concentrated hydrochloric acid were added. The supernatant solution analyzed for 1.22 mg of estrone. On heating the mixture for 5 minutes on the steam bath only 0.9845 mg. estrone were discovered. 50% of estrone had disappeared. The mixture was thoroughly extracted with ether and 1.786 mg. of estrone were recovered (95%). This observation indicates, that estrone can be removed from solution by acidified zinc and that it can be recovered from adsorption by ether extraction. This finding might be of value for commercial extraction of hydrolyzed urines.

30. The reduction of estrone with zinc and hydrochloric acid.

Estrone (3 mg.) was dissolved in ethanol (4 ml.) and diluted with 100 ml. of water. 10 volumes % concentrated hydrochloric acid and 4% zinc were added and the mixture was refluxed for 10 minutes. The estrogens were extracted with ether (3x40 ml.), and the extracts were washed neutral and taken to dryness. The residue was treated with Girard's reagent "T" and the non-ketonic fraction was chromatographed on alumina. The ketonic fraction did not contain any estrone. From the chromatogram (No.30) 75% of the recovered estrogenic material was obtained as desoxyestrone (CXXXIV). 17% was identified as α-estradiol, 3% was a mixture of α- and β-estradiol and 5% were not identified.

Chromatogram No.30.

ketonic	---		
non-ketonic			
1.Bz.100%	---	oil	
2. " 2%acetone	---	oil	
3. " 5 "	0.4817mg.	135-137°C	
4. " 7½ "	1.233 "	"	desoxyestrone
5. "10 "	0.2585 "	123-125°C	
6. "15 "	0.1456 "	oil	
7. "20 "	0.3239 "	178°C	α-estradiol
8.Ether 100%	0.0864 "	176°C,219-233°C	α,β-estradiol
9.CH_3OH "	---	oil	

31. Clemmensen reduction of estrone.

Estrone (2.7 mg.) was dissolved in 5 ml. acetic acid. Amalgamated mossy zinc (prepared by leaving zinc in a 5% solution of mercurous chloride in water for one hour and decanting the solution) and concentrated hydrochloric acid (5 ml.) were added, and the mixture was heated for two hours on the steam bath.The solution was extracted with ether(3x20 ml.) after ten-fold dilution with water.The extracts were washed and taken to dryness. On separation with Girard's reagent "T", 90% of the material was found in the ketonic fraction and identified as estrone.The non-ketonic material was chromatographed (No.31), and crystals of desoxyestrone were obtained, which were used for a mixture melting point determination with the crystals obtained in section 30.

32. Reduction of estrone by zinc and hydrochloric acid.II.

To obtain larger quantities of desoxyestrone, 30 mg. estrone were reduced with 4% zinc and 10 volumes % concentrated

hydrochloric acid in 100 ml. of water. After 10 minutes the reaction product was extracted with ether, purified and separated into ketonic and non-ketonic material with Girard's reagent "T". 15 mg. estrone were recovered. From the chromatogram of the non-ketonic fraction (no.32) 5 mg. of α-estradiol was recovered. 0.3 mg. of an oily fraction was obtained which possessed the characteristic estrogenic spectrum. Possibly impure desoxyestrone. ß-estradiol could not be discovered.

Chromatogram No. 31.

ketonic	2.403 mg. Estrone	
non-ketonic		
1. Bz. 100%	---	
2. " 2% acetone	---	
3. " 5 "	0.293 "	Desoxyestrone
4. " 7½ "		"
5. "10 "		"
6. "15 "	---	
7. "20 "	---	
8. "20 "	---	
9. Ether 100%	---	
10. CH_3OH "	---	

Chromatogram No. 32.

ketonic	15.423 mg.		
non-ketonic			
1. Bz. 100%	---	oil	
2. " 2%acetone	---	"	
3. " 5 "	---	"	
4. " 7½ "	0.1482"	"	
5. " 10 "	0.1525"	"	
6. " 15 "	0.9045"	176°C	α-estradiol
7. " 20 "	3.953 "	"	"
8. Ether 100%	---	oil	
9. CH_3OH "	---	"	

33. Clemmensen reduction of estrone. II.

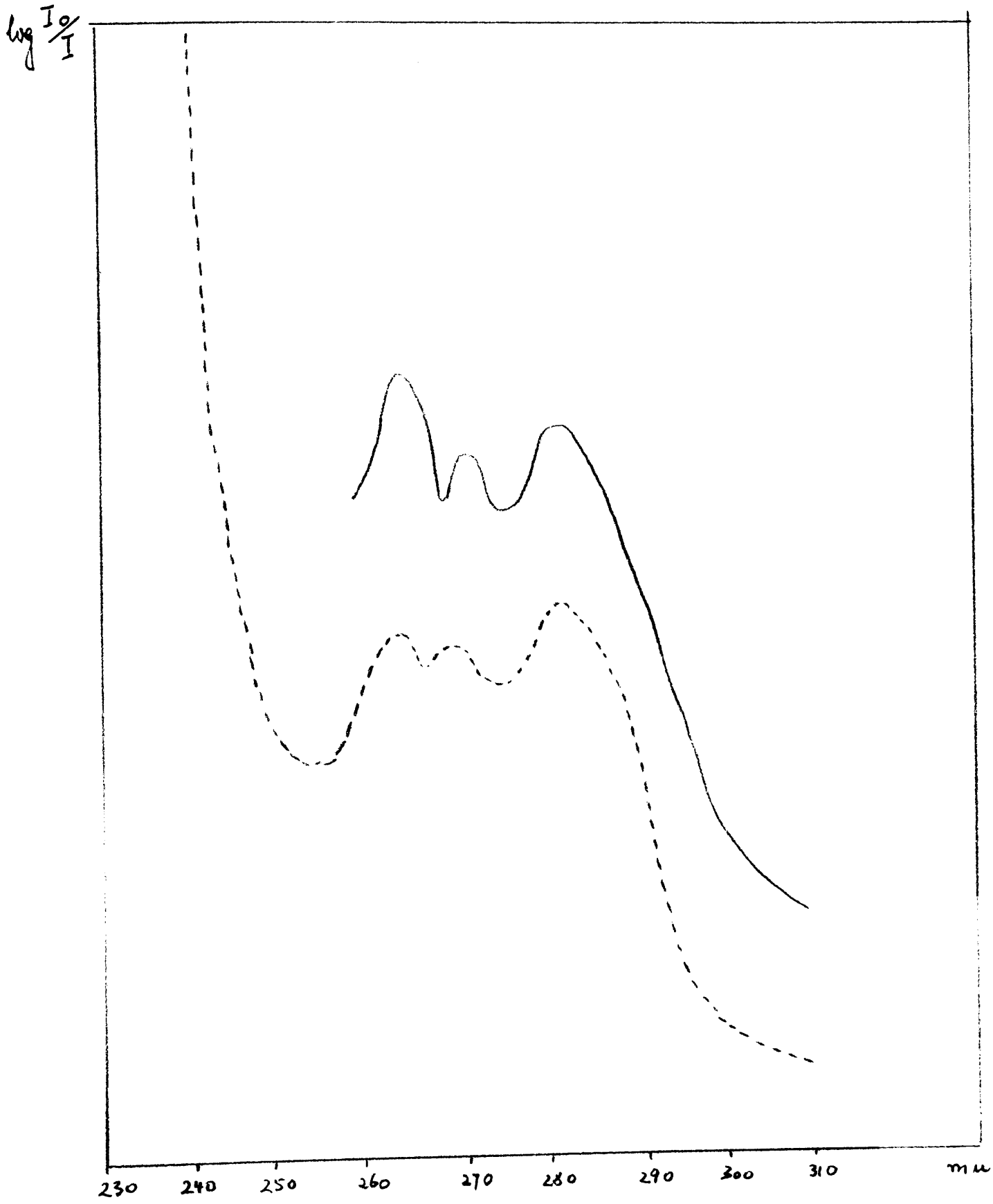
43.6 mg. estrone were reduced with amalgamated zinc wool(as previously described). After heating for 16 hours on the steam bath, the cooled solution was diluted with 20 volumes of water, extracted with ether(3x60 ml.), and the ether extracts were washed neutral and taken to dryness. The residue was treated with Girard's reagent "T". 50% was recovered as estrone. The non-ketonic fraction on chromatography(No.33) yielded 5.1 mg. desoxyestrone. Other crystalline fractions, possessing the 280 mμ absorption band, were eluted but could not be identified. With 100% ether crystals were eluted, m.p. 99-100°C, which showed three absorption bands in their spectrum. This spectrum was of interest, since a similar spectrum had been observed in a weak phenolic fraction of acid treated pregnancy urine. (Graph on page 186.)

Chromatogram 33.

ketonic	20.1	mg.		
non-ketonic				
1. Bz. 100%	----	"	oil	
2. " 2%acetone	0.130	"	oil	
3. " 5 "	5.103	"	133-136°C	Desoxyestrone
4. " 7½ "	6.167	"	123-25,197°C	?
5. " 10 "	2.402	"	192-197°C	?
6. " 15 "	1.780	"	oil	
7. " 20 "	1.574	"	99-100°C λ_{max} 264,270,280 mμ	?
8. Ether 100%	4.683	"	oil	

The ketonic fraction was subjected to another Clemmensen reduction. 14.1 mg. starting material was recovered. In the non-ketonic fraction 1.18 mg. desoxyestrone was isolated.

Absorption spectrum of Clemmensen reduction product of estrone(---) and of phenolic urine fraction(——).



34. The action of faecal bacteria on estrone.

Estrone (47 mg.) was dissolved in ethanol (5 ml.) and added to a suspension of 100 g. rat faeces in saline. The mixture was incubated at 37°C for one week. The estrogens were extracted with ether(6x30 ml.). 20.63 mg. material, possessing the 280 mμ absorption band, was obtained. The estrogens were separated into ketonic and non-ketonic phenols and chromatographed (No. 34 K and 34 N). 7.7 mg. estrone was eluted together with 2.2 mg. ketonic oils possessing the 280 mμ maximum. In the non-ketonic fraction 4.6 mg. α-estradiol and 1.4mg. β-estradiol were eluted and 4.6 mg. oily fractions, also analyzing as estrogens were isolated.

Chromatogram 34 N

1. Bz.	100%	----	oil	
2. "	2% acetone	----	oil	
3. "	4 "	----	130°C	
4. "	10 "	----	oil	
5. "	15 "	4.670 mg.	175-180°C	α-estradiol
6. "	20 "	2.836 "	oil	?
7. Ether	100%	1.439 "	226-228°C	β-estradiol
8. CH_3OH	"	1.827 "	oil	?

Chromatogram 34 K

1. Bz.	100%	----	oil	
2. "	2% acetone	----	oil	
3. "	4 "	4.769 mg.	249-251°C	estrone
4. "	10 "	2.955 "	"	"
5. "	15 "	0.675 "		?
6. "	20 "	0.211 "	oil	?
7. Ether	100%	0.086 "	oil	?
8. CH_3OH	"	1.223 "	oil	?

Summary and Conclusion of Part 3.

A search for the hypothetical estrogen oxidation product, which can be reactivated by zinc and hydrochloric acid treatment (according to Smith and Smith), was made on pregnancy urine. None of the proposed oxidation products could be discovered on thorough investigation of all fractions of the urine extracts. After the second hydrolytic treatment in the presence of 4% zinc, large quantities of estriol were extracted from the aqueous phase. As Smith had accepted his method of hydrolysis to give complete liberation of the estrogens present, the additional quantity of estriol liberated could not be accounted for, giving rise to the concept of the $T_{Zn/To}$ ratio. On the basis of incomplete hydrolysis of estriol glucuronate alone, a ratio of 2 to 3 was obtained in all experiments. This additional estriol was also obtained on second hydrolysis (3 hours) without zinc in the autoclave or under a stream of nitrogen. Without protection from atmospheric oxygen the yield of estriol was considerably smaller. These observations were confirmed by hydrolysis experiments on estriol glucuronate and evidence for the following explanations was obtained.

After 10-minutes hydrolytic treatment of the urine, the estrogens were extracted with ether. When the aqueous phase was subjected to another hydrolytic treatment, no further quantities of estrogens were obtained, and it was assumed

that hydrolysis had been completed. However, the ether, present in solution in the urine, is oxidized in the presence of atmospheric oxygen to ether peroxides, which destroy the estrogenic material. The hydrogen atmosphere, produced by the action of zinc on hydrochloric acid, prevents this destruction. Besides its protective action, zinc treatment has a number of disadvantages.

It was observed that zinc would adsorb large quantities of estrone and estriol, which could only be recovered on thorough extraction. Secondly, the reduction of estrone by zinc and hydrochloric acid, which uniformly increased the biological activity of the estrone present five times in Smith and Smith's investigations, was found to give varying results. In concentrations, comparable to those in pregnancy urine, reduction of estrone by Smith's method gave rise to desoxyestrone (90%), while the remaining 10% consisted predominantly of α-estradiol with small amounts of β-estradiol. The biological activity of desoxyestrone has not been determined, but the compound is not soluble in N sodium hydroxide solution and will therefore not be estimated in the phenolic fraction. In the presence of larger quantities of estrone, reduction is very incomplete, leaving part as estrone, while most of it is recovered as α-estradiol with only traces of β-estradiol. Variations in the concentration of estrone must, therefore, lead to great differences in activity on biological assay depending on the product formed.

No evidence for the existence of an inactive estrogen oxidation product was obtained. This observation is in line with the findings of Cantarov et al., that the liver does not destroy the estrogens, but removes them from circulation secreting them into the bile. With the bile the estrogens pass into the intestine and are reabsorbed, establishing an entero-hepatic circulation. Whether all estrogens are slowly excreted in the urine as conjugates or can be inactivated by the faecal bacterial flora, when passing through the intestine, was investigated.

On incubation of rat faeces with estrone in saline, it was observed, that 37% of the recovered estrogens was α-estradiol, 7.5% was identified as β-estradiol, while 40% was unchanged estrone. As 50% of the estrogenic material was recovered, the probability of bacterial inactivation of the estrogens in the intestinal tract appears insignificant.

A quantitative recovery of the estrogens administered can be expected, when the urine is collected over a longer period, and when hydrolysis of the conjugates is carried out with all necessary precautions.

References.

The discovery of estrogens in urine.

1. Loewe et al. Klin.Woch.,4,1407,1925.
2. Aschheim " Klin.Woch.,6,1322,1927.
3. Zondek " Klin.Woch.,7, 485,1928.
4. Doisy " A.J.Physio.,90,329,1929.
5. Butenandt " Naturwis., 17,878, 1929.
6. Marrian " B.J.,24, 1021, 1930.
7. Doisy " J.B.C., 86,499,1930.
8. Butenandth" Z.P.C.,199,243,1931.
9. Smith " J.B.C.,130,431,1939.
10. Hirshman " J.B.C.,122,303,1937.
11. Doisy " J.B.C.,115,435,1936.
" J.B.C.,126,181,1938.

Metabolism of estrogens: Interconversions.

12. Doisy et al. Ann.Int.Med.,11,267,1937.
13. Pincus Vit. & Horm.1,233,1943.
14. Dorfman " Endo., 36,347,1945.
15. Dorfman " Science,91,388,1940.
16. Heard " J.B.C.,141,329,1941.
17. Doisy " Fed.Proc.,1,202,1942.
18. Pincus " J.B.C.,144,569,1942.
19. Huffman " Science,100,312,1944.
20. Pincus " Endo.,31,507,1942.
21. Pincus " Arch.Bio.,2,317,'43.

22. Pincus et al. J.Gen.Physio.,20,879,1937.
23. Smith " A.J.Obst.& Gyn.,36,769,1938.
24. Smith " A.J.Physio.,121,98,1939.
25. Beall " B.J.,34, 1293,1940.
26. Pincus " Arch.Bio.,2, 317,1943.
27. Stroud " J. Endo.,1,201,1939.
28. Fish " J.B.C.,140,XL,1941.
29. Fish " J.B.C.,140,83,1941.
30. Fish " J.B.C.,143,15,1942.
31. Heard " J.B.C.,141,709,1941.
32. Pincus " Endo.,31, 507,1942.
33. Doisy Biol.Symp.IX,21,1942.

Estrogen inactivation by liver tissue.

34. Engel et al. Bio. Z.,292,434,1937.
35. Engel " Endo.,29,290,1941.
36. Zondek Proc.Soc.,46,276,1941.
37. Fels " Medicina B.A.,2,140,1942.
38. Golden " Proc.Soc.,39,361,1938.
39. Segaloff " Proc.Soc.,48,33,1941.
40. Engel " Endo.,35,70,1944.
41. Fels " Boll.,12,140,1944.
42. Lipshutz " Science,101,410,1945.
43. Silberstein Klin.Woch.,12,1693,1933.
44. v.Euler Bio.Z.,271,64,1934.
45. Zondek Skan.Arch.Phys.,70,133,1934.
46. Segaloff " Endo.,33,209,1943.

47. Biskind et al. Proc.Soc.,53,91,1943.
48. Heller " Endo.,26,619,1940.
49. Heller " A.J.Physio.,126,530,1939.
50. Biskind " Bul.J.Hop.H.,65,212,1939.
51. Biskind " Proc.Soc.,43,259,1940.
51. " Proc.Soc.,47,266,1941.
52. Biskind " Proc.Soc.,46,452,1941.
53. Israel " A.J.Med.Sci.,194,835,1937.
54. Pincus " Science,98,410,1943.
55. Heller " Science,99,145,1944.
56. Trombly " Canc.Res.,2,811,1942.

58. Heller " Endo.,32,64,1943.
59. Selye J.Pharm.,71,236,1941.
60. Selye J.Pharm.,80,386,1944.
61. Engel " Endo.,37,44,1945.
62. Engel " Ana.Soc.Bio.,2,17,1945.

Liver impairment and estrogen inactivation.

63. Talbot et al. Endo.,25,601,1939.
64. Glass " Endo.,27,749,1940.
65. Pincus " Endo.,27,838,1940.
66. Biskind " Endo.,31,101,1942.
67. Biskind " Endo.,30,819,1942.
68. Rakoff " Endo.,34,370,1944.
69. Shigley " Proc.Soc.,57,52,1944.
70. Schiller " Endo.,34,203,1944.

71. Unna et al. Proc. Soc., 55, 254, 1944.

72. Pincus " Endo., 36, 7, 1945.

73. Zondek " Clin. & Exp. Invest.

74. Cadden " J.B.C., 143, 605, 1942.

75. Böttiger " J. cell. Phys., 27, 65, 1946.

Effect of Vitamin B complex on estrogen inactivation.

76. Biskind " Science, 94, 462, 1941.

77. Biskind " Endo., 30, 819, 1942.

78. Biskind " Endo., 31, 109, 1942.

79. Biskind " Endo., 35, 187, 1944.

80. Singher " Endo., 35, 226, 1944.

81. Singher " J.B.C., 154, 79, 1944.

82. Segaloff " Endo., 34, 346, 1944.

83. Biskind " Science, 103, 441, 1946.

84. Drill " Fed. Proc., 5, p2, 24, 1946.

85. Gyorgyi " Proc. Soc., 57, 52, 1944.

86. Unna " Proc. Soc., 55, 254, 1944.

87. Gyorgyi " Proc. Soc., 60, 344, 1945.

88. Drill " Endo., 38, 300, 1946.

89. --- Nutri. Rev., 5, 26, 1947.

90. --- Nutri. Rev., 3, 308, 1945.

91. Hertz " Endo., 37, 1, 1945.

92. Koref " Endo., 38, 133, 1946.

Enzymatic inactivation of estrogens.

93. Miwa " Klin. Woch., 19, 854, 1941.

94. Mamoli Ber., 71, 2696, 1938.

95. Wettstein et al. Helv., 22, 250, 1939.
96. Raper " Physio. Rev., 8, 245, 1928.
97. Zondek " Lancet, 2, 356, 1934.
98. Zondek " Proc. Soc., 49, 629, 1942.
99. Zondek " Endo., 36, 291, 1945.
100. Graubart " Endo., 30, 265, 1942.
101. Westerfeld " B.J. 34, 51, 1940.
102. Figge " Proc. Nat. Ac. Sc., 27, 149, 41.
103. Pincus " Endo., 29, 262, 1940.
104. Pincus " A. J. Physio., 133, 298, 1941.
105. Pincus " Proc. Fed., 1, p.1., 31, 1942.

Oxidation in ring D of steroid molecule of estrogens.

106. Westerfeld et al. J.B.C., 140, CXXXVIII, 1941.
107. Westerfeld " J.B.C., 141, 177, 1941.
108. Comp. Rend., 139, 128, 1946.
109. Heard " J.B.C., 141, 329, 1941.
110. Smith " Proc. Soc., 36, 460, 1937
111. Smith " Endo., 28, 740, 1941.
112. Smith " J. clin. Endo., 1, 461, 1939.
113. Smith " Endo., 35, 146, 1944.
114. Smith " Proc. Soc., 59, 242, 1945.
115. Figge " Endo., 36, 178, 1945.
116. Marrian Harvey lec., 34, 37, 1938.

Entero-hepatic circulation of estrogens.

117. Longwell " J.B.C., 142, 757, 1942.
118. Cantarov " Endo., 31, 515, 1942.
119. Cantarov " Endo., 32, 368, 1943.

120. Cantarov et al. Proc.Soc.,52,256,1943.
121. Cantarov " Proc.Soc.,49,707,1942.
122. Cantarov " Endo.,33,309,1943.
123. Cantarov " Endo.,34(203)371,1944.
124. Cantarov " Endo,,30,1027,1942.
125. Cantarov " Science,101,558,1945.
126. Cantarov " Endo.,35,129,1944.
127. Eisen " Proc.Soc.,49,625,1942.
128. Szego " Endo.,32,368,1943.
129. Szego " J.B.C.151,599,1943.
130. Pashkis " Proc.Soc.,55,127,1944.
131. Marrione " Arch.Path.,32,39,1944.
132. Pearlman " Endo.,36,291,1945.
133. " Endo.,36,284,1945.
134. Joyce " J.Endo.,4,371,1946.

Estrogen excretion in faeces.

135. Dohrn et al. Klin.Woch.,7,943,1928.
136. Siebke " Zeit.Gynä.,54,1734,1930.
137. Frank " Proc.Soc.,29,1229,1932.
138. Dingemanse et al. A.J.Obst.& Gy.,33,1000,1937.
139. Dingemanse " Neder.Tijd.Gen.,84,3287,1940.
140. Zondek " Endo.,33,204,1943.

141. Marker " J.A.C.S.,60,1901,1938.
142. Heard " J.B.C.,135,801,1940.
143. Heard " J.B.C.,138,651,1941.
144. Doisy Fed.Proc.1,202,1942.

Estrogen conjugation.

146. Fishman et al. — J.B.C., 127, 367, 1939.

147. Fishman " — J.B.C., 131, 225, 1939.

148. Fishman " — J.B.C., 136, 228, 1940.

149. Fishman " — J.B.C., 152, 487, 1944.

150. Daudel " — Experiencia, 2, 107, 1946.

151. Collip " — Endo., 18, 71, 1931.

152. Collip " — J.B.C., 97, XVII, 1932.

153. Cohen " — B.J., 29, 1577, 1935.

154. Cohen " — Lancet, 1, 674, 1935.

155. Cohen " — B.J., 30, 57, 1936.

156. Cohen " — B.J., 30, 2250, 1936.

157. Venning — McGill Med. J., 12, 301, 1943.

158. Marrian " — Coldspring H. Sym. V. 16, 1937

159. Marrian " — Ergebnisse d. Vitamin & Hormon Forschung, 1, 441, 1938.

160. Marrian " — J.B.C., 126, 663, 1938.

161. Marrian " — Proc. Soc., 35, 222, 1936.

162. Butenandt — Z.P.C., 259, 222, 1939.

163. Jensen — Rev. Can. Bio., 4, 535, 1945.

164. Smith " — A. J. Physio., 100, 553, 1932.

165. Smith " — A. J. Physio., 98, 578, 1931.

166. Smith " — A. J. Physio., 112, 340, 1935.

167. Smith " — New Eng. J. Med., 215, 908, 1936.

168. Smith " — A. J. Physio., 121, 98, 1937.

169. Smith " — A. J. Obst. & Gy., 36, 769, 1938.

170. Smith " — A. J. Obst. & Gy., 39, 405, 1940.

171. Smith et al. Proc.Soc.,44,104,1940.

172. Smith " Proc.Soc.,38,713,1938.

173. Smith " Endo.,25,509,1939.

174. Smith " A.J.Obst & Gy.,39,505,1940.

175. Smith " J.clin.Endo.,1,461,470,477,1941.

176. Smith " A.J.Obst.& Gy.,44,455,606,1942.

177. Smith " A.J.Obst.& Gy.,45,15,23,43,1943.

178. Smith " A.J.Med.Sci.,208,25,1944.

179. Smith " J.clin.Endo.,6,483,1946.

180. Viegas Brasil Med.,52,877,1938.

181. Durupt Gyné et Obst.,37,183, 1938.

Fractionation of estrogens.

182. Marrian et al. B.J.,28,1603,1934.

183. Dingemanse " B.J.,31,500,1937.

184. Smith " Endo.,25,509,1939.

185. Bachman " J.B.C.,138,689,1941.

186. Mather " J.B.C.,144,617,1942.

187. Longwell " J.B.C.,142,757,1942.

188. Pearlman " Endo.,31,507,1942.

189. Pearlman " J.B.C.,147,379,1943.

190. Taylor " Canc.Res.,3,180,1943.

191. Girard " Helv.,19,1095,1936.

192. Westerfeld " J.B.C.,126,181,1938.

193. Whitman " J.B.C.,118,788,1937.

195. Huffman " J.B.C.,133,567,1940.

196. Huffman " J.B.C.,134,591,1940.

197. Pincus " Endo.,31,507,1942.

198. Pincus et al. J.B.C., 147, 382, 1943.

199. Pearlman Arch.Bio., 4, 97, 1944.

200. Duschinski " Bul.Soc.Chem.Bio., 17, 1534, 1935.

201. Hirschman " J.B.C., 122, 303, 1937.

203. Bauman " Endo., 27, 664, 1940.

204. Bauman " Endo., 36, 391, 1945.

205. Stimmel " J.B.C., 153, 327, 1944.

206. Stimmel " J.B.C., 162, 99, 1946.

207. Stimmel " J.B.C., 165, 73, 1946.

208. Fieser Phenanthrene Prod., 187, 1937.

209. Wintersteiner J.B.C., 130, 35, 1939.

210. Wintersteiner J.B.C., 132, 605, 1939.

211. Wintersteiner J.B.C., 133, 219, 1939.

212. Wintersteiner J.B.C., 141, 597, 1941.

213. Wintersteiner J.B.C., 137, 785, 1941.

214. Huffman et al. J.B.C., 134, 591, 1940.

215. Huffman " J.A.C.S., 64, 2235, 1942.

216. Pincus unpublished com.

217. Huffman " J.A.C.S., 66, 150, 1944.

Hydrolysis of urinary estrogens.

218. Laqueour et al. Naturwis., 22, 190, 1934.

219. Doisy " J.B.C., 79, 53, 1928.

220. Siebke " Zeit.Gynä., 54, 1601, 1930.

221. Funk " B.J., 24, 1678, 1930.

222. Kemp " Endokri., 13, 156, 1933.

223. Dingemanse et al. — Naturwis., 22, 190, 1934.

224. Dingemanse " — B.J., 31, 500, 1937.

225. Kurzrok " — A.J.Obst.& Gy., 23, 689, 1932.

226. Marrian — Physio.Rev., 13, 185, 1933.

227. Cohen " — B.J., 28, 1603, 1934.

228. Patterson " — Bri.Med.J. ii, 522, 1937.

229. Zondek — Nature, 133, 209, 1934.

230. Marrian " — B.J., 29, 1577, 1934.

231. Beall " — B.J., 30, 577, 1936.

232. Beall " — J.S.C.I., 53, 309 T, 1934.

233. Koch " — Proc.Soc., 33, 440, 1936.

234. Smith " — Proc.Soc., 36, 460, 1937.

235. Browne " — J.B.C., 120, 225, 1937.

236. Schmulowitz " — J.Lab.& Cli.Med., 22, 1037, 1937.

237. Schmulowitz " — J.Lab.& Cli.Med., 23, 633, 1938.

238. Peterson " — J.B.C., 119, 185, 1937.

239. Heard " — J.B.C., 130, 579, 1939.

240. Dingemanse " — B.J., 32, 65, 1938.

241. Smith " — A.J.Physio., 121, 98, 1938.

242. McCullough " — Endo., 22, 120, 1938.

243. Hain " — Quart.J.Exp.Phy., 29, 139, 1939.

244. Cherry " — Proc.Soc., 40, 688, 1939.

245. Palmer " — J.Lab.& Cli.Med., 24, 643, 1939.

246. Callow " — J.Endo., 1, 76, 1939.

247. Smith " — Endo., 25, 509, 1939.

248. Pearlman " — J.B.C., 147, 379, 1943.

249. Talbot " — J.B.C., 148, 593, 1943.

250. Friedgood " — Chem.& Phys.of Hormones, 195, 1944.

251. Leiboff et al. J.Lab.& Cli.Med.,24,178,1938.
252. Doisy " Fed.Proc.,1,202,1942.
253. Doisy " Endo.,30,933,1942.
254.Varanget " Compt.Rend.,136,287,1942.
255. Mallow " Arch.Gynä.,172,601,1942.
256. Watts " A.J.Obst.& Gy.,46,183,1943.

Reduction of estrone.

257. Girard et al. Compt.Rend.,112,964,1933.
258. David " Arch.Int.Phar.,51,137,1935.
259. Danielli " B.J.,27,311,1933.
260. Westerfeld J.B.C.,126,181,1938.
261. Butenandt Z.P.C.,248,129,1937.
262. Wintersteiner J.A.C.S.,59,765,768,1937.
263. Dirscherl Z.P.C.,239,53,1936.
264. Marks " J.A.C.S.,59,768,1937.
265. Schwenk " Naturwis.,21,777,1933.
266. Marker " J.A.C.S.,60,2927,1938.
267. Wintersteiner J.B.C.,118,789.1937.
268. Butenandt Z.P.C.,208,149,1932
269. Butenandt Z.P.C.,248,129,1937.
271. Marrian J.S.C.I.,51,277 T,1932.
272. Butenandt Z.P.C.,223,147,1934.
273.Butenandt Z.P.C.,191,127,1930.
274. Butenandt Z.P.C.,208,171,1932.
275. Butenandt Nature,130,238,1932.
276. Doisy etal. J.B.C.,99,327,1933.
277. Wintersteiner et al. J.B.C.,130,35,1939.

Bioassay of estrogens.

Bioassay of estrogens.

278. Doisy et al. J.A.M.A., 81, 819, 1923.

279. Doisy " Proc. Soc., 32, 707, 1935.

280. Fluhmann " Endo., 18, 705, 1934.

281. Astwood " Endo., 23, 25, 1938.

282. Dorfman " Proc. Soc., 45, 594, 1940.

283. Durupt Comp. Rend., 126, 382, 1938.

284. Gustavson A.J.Obst.& Gy. 35, 115, 1938.

285. Pincus " Canc. Res., 1, 970, 1941.

286. Koch " Endo., 31, 162, 1942.

287. Westerfeld J.B.C., 126, 181, 1938.

288. Pugsley " Endo., 33, 48, 1943.

289. Pugsley " Fed. Proc., 5, p. 2, 65, 1946.

290. Pugsley " Fed. Proc., 5, p. 2. 150, 1946.

291. Emmens J. Physio., 94, 22 P, 1939.

292. Marker J.A.C.S., 59, 1367, 1937.

Colorimetric estimation of estrogens.

293. Schwenk " Bio. Z., 259, 240, 1933.

295. Cuboni " Klin. Woch., 13, 302, 1934.

296. Häussler Helv., 17, 531, 1934.

297. David Acta brev. Neerl., 4, 64, 1934.

298. Schmulovitz J.B.C., 116, 415, 1936.

299. Cartland J.B.C., 109, 213, 1935.

300. Gerngross Z. ang. chem. 45, 775, 1932.

301. Sobotka J.B.C., 124, 693, 1938.

302. Bachmann et al. J.B.C., 131, 455, 463, 1939.

303. Zimmermann Klin. Woch., 17, 1103, 1938.

304. Kober Bio. Z., 239, 209, 1931.

305. Marrian et al. B.J.,28,1603,1934.

306. Pincus " J.B.C.,116,253,1936.

308. Venning " J.B.C.,120,225,1937

309. Chevallier " Bul.Acad.Med.,114,171,1935.

310. Kober B.J.,32,357,1938

311. Bachman " J.B.C.,138,689,691,1941.

312. Szego " Proc.Soc.,43,263,1940.

313. Szego " J.B.C.,151,587,1943.

314. Joyce " Bul.Soc.Chim.Bio.,25,301,1943.

315. Joyce " Bul.Soc.Chim.Bio.,28,363,1946.

Ultraviolet spectrophotometry of estrogens.

316. Butenandt Z.P.C.,191,143,1930.

317. Euler at al. Z.P.C.,208,129,1932.

318. Euler " Ark.Kemi.11,5,1932.

319. Morton Appl.of absorpt.spectr.to
Vitamins & Hormones,205,217,1934.

320. Brody

321. Loofbourov Vit.& Hormones,1,109,1943.

322. Hogness J.B.C.,120,239,1937.

323. Campbell Nature,153,745,1944.

324. Callow et al. B.J.,30,906,1936.

325. John " Z.P.C.,250,18,1937.

326. Slosse " Comp.Rend.,130,822,1939.

327. Florence Bul.Soc.Chim.Bio.,28,657,1946.

328. Allen " J.A.M.A.,85,94,1925.

329. Doisy " Proc.Soc.,24,216,1926.

330. Laporta " Arch.di.Sc.Bio.,24,452,1938.

331. Butenandt — Ber., 75, 1931, 1942.

332. Figger et al. — Bul. School Med. Maryl., 27, 159, 1942.

Estrogens in blood.

333. Rakoff et al. — A. J. Obst. & Gy., 38, 371, 1939.

334. Albrieux " — J. Clin. Endo., 1, 893, 1941.

335. Frank " — A. J. Obst. & Gy., 43, 865, 1941.

336. Mühlbock " — Z. P. C., 250, 139, 1937.

337. Mühlbock " — Lancet, 634, 1939.

338. Mühlbock " — Lancet, 236, 1939.

339. Rakoff " — A. J. Obst. & Gy., 46, 856, 1943.

340. Mühlbock — Acta brev. Neerl., 10, 1, 1945.

341. Doisy " — J. B. C., 115, 435, 1936.

342. Cantarov " — A. J. Obst. & Gy., 46, 856, 1943.

343. Szego " — Fed. Proc., 5, p. 2, 103, 1946.

344. Szego " — Endo., 39, 183, 1946.

345. Szego " — Proc. Soc., 61, 161, 1946.

346. Böttiger — J. Cell Physio., 28, 1946.

Urine estrogens during the menstrual cycle.

347. Simonett " — Presse méd., 51, 8, 91, 1943.

348. d'Amour — A. J. Obst. & Gy., 40, 958, 1941.

349. d'Amour " — J. Clin. Endo., 1, 433, 1941.

350. Mayer — Endo., 27, 867, 1940.

351. Gustavson " — A. J. Obst. & Gy., 35, 115, 1938.

352. Yerby — Proc. Soc., 36, 496, 1937.

353. Werner — J. Clin. Inv., 20, 21, 1941.

354. " — J. Clin. Inv., 22, 395, 1943.

Bioassay of Progesterone.

355. Corner et al. — A.J.Physio., 88, 326, 1929.

356. Allen " — A.J.Physio., 92, 174, 1930.

357. Clauberg " — Zeit.Gynä., 54, 2575, 1930.

358. McPhail " — J.Physio., 83, 145, 1934.

359. McGinty " — Endo., 24, 829, 1939.

360. Haskins " — J.Clin.Endo., 1, 65, 1941.

361. de Fremery — Arch.ges.Phys., 231, 341, 1932.

362. Ehrhardt " — Endokri., 16, 1, 1935.

363. v.Bloch — Endo., 20, 307, 1936.

364. de Alleve — Rev.Soc.Arg.Bio., 16, 755, 1940.

365. Hoffmann " — Zent.Gynä., 66, 1145, 1942.

366. Hoffmann " — Zent.Gynä., 66, 292, 1942.

367. Hoffmann " — Zent.Gynä., 65, 2014, 1941.

368. Hoffmann " — Zent.Gynä., 67, 1082, 1943.

Physical methods of Progesterone determinations.

369. Heyrovsky — Coll Czech.Chem.Com., 11, 98, 1939.

370. Wolfe " — J.B.C., 136, 653, 1940.

371. Wolfe " — J.Y.C.S., 63, 582, 1941.

372. Menschnick — Ann., 495, 225, 1932.

373. Reynolds " — Endo., 31, 147, 1942.

374. Woodward — J.A.C.S., 63, 1123, 1941; 2727, 1941.

375. Woodward — J.A.C.S., 64, 72, 76, 1942.

376. Allen " — J.B.C., 98591, 1932.

377. Allen " — A.J.Pharm., 116, 291, 1944.

Lipid extraction from blood.

378. Flosdorf " — J.Immunol., 50, 21, 1945.

379. Chargaff " — J.B.C., 136, 257, 1940.

380. Macheboeuf et al. Comp.Rend.,129,1181,1184,

381. Macheboeuf " BulSoc.Chim.Bio.,14,1168,

382. Macheboeuf " Bul.Soc.Chim.Bio.,11,268,

383. Macheboeuf " Bul.Soc.Chim.Bio.,11,485,

384. Tayran " Comp.Rend., ,239,1943.

385. McFarlane " Nature,149,439,

386. Chargaff " Science,99,147,

387. Delsal " Bul.Soc.Chim.Bio.,26,99,1944.

388. Pincus " Endo.,29,413,1941.

The part-synthesis of 12-keto-DCA.

389. Reichstein et al. Helv.,26,721,1943.

390. Gallagher " OSRD.confidential communication

391. Kendall " OSRD. " "

392. Sainte-Marie M.Sc.Thesis,McGill,April 1944.

393. Reichstein " Helv.,26,511,1943.

394. Long " Endo.,26,309,1940.

395. Ingle " Proc.Soc.,44,176,1940.

396. Kendall " Proc.Mayo.,15,565,294,1940.

397. Jensen " A.J.Physio.,128,270,1940.

398. Jensen " J.B.C.,135,511,1940.

399. Everse " Acta brev.Neerl.,2,152,1942.

400. Kendall " Endo.,31,573,1942.

401. Ingle " Endo.,30,246,1942.

402. Julian " U.S.P.2341557.

403. Reichstein " Helv.,20,1164,1937.

404. Butenandt " Ber.,67,1901,1934.68,2091,1935.

405. Butenandt " Ber.,72,1617,1939.73,451,1940.

406. Ruzicka " Helv.,21,866,1938.

407. Fieser — Exp. in Org. Chem. 361, 363, 381.

408. Blatt — Org. Synth. Coll. Vol. 2, 165, 461.

409. Gallagher et al. — J.B.C., 162, 511, 1946.

410. Wallis " — J.B.C., 162, 641, 1946.

411. Sarrett " — J.B.C., 162, 601, 1946.

412. Wintersteiner — J.B.C., 162, 725, 1946.

Preparation of 9:11-lithocholenic acid.

413. Reichstein et al. — Helv., 25, 797, 1942.

414. Kaziro " — Z.P.C., 249, 220, 1937.

415. Wallis " — J.B.C., 126, 633, 1938.

416. Schwenk " — J.A.C.S., 65, 549, 1943.

417. Reichstein " — Helv., 25, 797, 1942.

418. Wieland " — Z.P.C., 211, 261, 1932.

419. Gallagher " — J.B.C., 162, 521, 1946.

420. Schwenk — Personal communication

421. Wintersteiner " — OSRD. confidential com.

422. Kendall " — J.B.C., 162, 571, 1946.

423. Reichstein " — Helv., 26, 536, 1943.

424. Reichstein " — Helv., 26, 586, 1943.

425. Chravlowitz " — J.A.C.S., 62, 318, 1940.

426. Reichstein " — Helv., 25, 878, 1942.

427. Kendall " — J.B.C., 162, 555, 1946

428. Schwenk " — J.A.C.S., 65, 549, 1943.

429. Wallis " — J.A.C.S., 62, 318, 1940.

430. Wintersteiner " — J.A.C.S., 61, 1998, 1939.

431. Wintersteiner " — J.A.C.S., 62, 203, 1940.

432. Wallis " — J.A.C.S., 62, 219, 1940.

433. Ruzicka " — Helv., 18, 668, 1935.

434. Allen et al. J.A.C.S.,52,2957,1930.

435. Brady " J.C.S.,757,1931.

436. Brockmann " Ber.,74,B,73,1941.

437. Schönheimer J.B.C.,114,567,1936.

Nitration.

438. Michael & Carlson J.Org.Chem.,4,169,1939.

439. Michael & Carlson J.Org.Chem.,5,1,14,1940.

440. Heilbron J.C.S.,104,1938.

441. Wallis et al. OSRD.confidential com.

442. Montignic " Bul.Soc.Chim.,1,291,1934.

443. Riebsomer " Chem.Rev.,36,197,1945.

444. Schmidt " Ber.,35,2323,1902.

445. Seidorenko Chem.Zent.,I,399,1907.

446. Wallach " Ann.,241,291,

447. Wallach " Ann.,332,305,1904.

448. Windaus " Ber.,52,162,1919.53,488,1920.

449. Nightingale J.A.C.S.,66,352,1944.

450. Haas " Chem.Rev.,32,412,1943.

451. Bouvault " Bul.Soc.Chim.,29,517,1903.

452. Houben Methoden d.Org.Chemie.

Sidechain degradation.

453. Ruzicka et al. Helv.,17,1395,1389,1934.

454. Ruzicka " Helv.,18,61,430,668,1483,1935.

455. Ruzicka " Helv.,20,1283,1291,1937.

456. Reichstein " Helv.,26,1328,2102,1943.

457. Barbier " Comp.Rend.,156,1,443,1913.

458. Wieland " Z.P.C.,161,1,80,1926.

459. Kendall " OSRD.confidential com.

460. Hoehn & Mason — J.A.C.S., 60, 1493, 1938.

461. Heard et al. — OSRD. confidential com.

462. Heard et al. — unpublished observations.

463. Reichstein et al. — Helv., 20, 1, 3, 949, 1040, 1937.

464. Jacobson — J.A.C.S., 66, 662, 1944.

465. Maystre et al. — Helv., 27, 1815, 1944. 28, 1252, 1945.

466. Ehrenstein — J.Org.Chem., 5, 660, 1940.

Bromination.

467. Gilman — Org. Synth., Col. Vol. I., 108.

468. Schmidt — Org. Chemistry, 177.

469. Richter — Org. Chemistry, I, 331.

470. Hickinbottom — Reactions of org. comp. 209.

471. Houben — Methoden d. Org. Chem. 1154, 1165

472. Zelinski — Ber., 20, 2026,

473. Vollhart — Ann., 242, 141,

474. Wallis et al. — J.B.C., 162, 695, 1946.

475. Fleischer " — OSRD. confidential com.

476. Gallagher " — J.B.C., 162, 549, 1946.

477. Marker — J.A.C.S., 64, 817, 822, 1276, 2089, 1942.

478. Marker — J.A.C.S., 64, 1128, 2093, 1942.

479. Wintersteiner — J.A.C.S., 62, 200, 1940.

480. Karrer et al. — Helv., 573, 29, 1946.

Abbreviations.

Acta brev.Neerl.	Acta brevia Neerlandica.
A.J.Med.Sci.	American Journal of the Medical Sciences.
A.J.Obst.& Gy.	American Journal of Obstetrics and Gynecology.
A.J.Phy.	American Journal of Physiology.
Ann.	Liebig's Annalen der organischen Chemie.
Ann.Int.Med.	Annals of Internal Medicine.
Arch.Bio.	Archives of Biochemistry.
Arch.Gynä.	Archives für Gynäkologie.
Arch.Int.Pharm.	Archives of International Pharmacology.
Arch.Path.	Archives of Pathology.
Ber.	Berichte der Deutschen Chemischen Gesellschaft.
B.J.	Biochemical Journal.
Biol.Symp.	Biological Symposia.
B.Z.	Biochemische Zeitschrift.
Bri.Med.J.	British Medical Journal.
Bul.Acad.Med.	Bulletin of the Academy of Medicine.
Bul.J.Hop.H.	Bulletin of John Hopkins Hospital.
Bul.Sch.Med.Maryl.	Bulletin of the School of Medicine, Maryland.
Bul.Soc.Chim.	Bulletin de la Société de Chimie.
Bul.Soc.Chim.Bio.	Bulletin de la Société de Chimie Biologique.
Canc.Res.	Cancer Research.
Chem.Rev.	Chemical Reviews.
Chem.Zent.	Chemisches Zentralblatt.
Coldspring H.Symp.	Coldspring Harbour Symposium
Comp.Rend.	Comptes Rendus des Séances de la Société de Biologie.
Endo.	Endocrinology.
Endokri.	Endokrinologie.
Fed.Proc.	Federation of the American Society, Proceedings.
Harvey lec.	Harvey Lectures.
Helv.	Helvetica Chimica Acta.
JACS.	Journal of the American Chemical Society.
J.A.M.A.	Journal of the American Medical Association.
J.C.S.	Journal of the Chemical Society.
J.cell.Phy.	Journal of Cellular Physiology.
J.clin.Endo.	Journal of clinical Endocrinology.
J.clin.Inves.	Journal of clinical Investigation.