SOME ASPECTS OF THE BIOCHEMISTRY OF THE STEROID HORMONES.

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of

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by

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GENERAL INTRODUCTION.

This work was originally undertaken in order to elucidate certain features of the structure of the substance known as "sodium oestriol monoglucuronidate"[x] isolated by Cohen, Marrian and Odell (1936), and to investigate its purity. The work was beset by difficulties and delays, particularly the low yields in the preparation of purified NaOG. These may be attributed in part to the difficulty in obtaining fresh supplies of genuine late pregnancy urine from the busy wards of the Maternity Hospital. Consequently attention was diverted to other problems which could be investigated while supplies of NaOG accumulated.

Through the kindness and co-operation of Dr. S.J. Folley of the National Institute for Research in Dairying an opportunity was given for the examination of the urine of goats receiving large doses of progesterone. This investigation failed to yield results of importance in steroid metabolism but led to the isolation and identification of p-ethylphenyl

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x This will be referred to as NaOG in this thesis. HOG will be used as an abbreviation for oestriol monoglucuronidic acid.

sulphuric acid which has not been previously obtained from animal sources.

More recently, problems of the in vitro metabolism of pregnanediol by surviving tissue and enzyme preparations have been studied. This work has produced promising results, and the problems of the chemistry of oestriol glucuronide have been shelved until more suitable methods are available for the production of reasonable quantities of this substance. The results of the in vitro metabolism experiments form the most important part of this thesis. The work on the chemistry of oestriol glucuronide is described next, and finally the results of the examination of the goat urines are recorded.

Following the proposals of Fieser and Fieser (1949) the name dehydroepiandrosterone is used for dehydroisoandrosterone, and oestradiol-17β is used to denote the more physiologically active 17 hydroxy isomer. The term pregnanediol is generally used for pregnane-3α:20α-diol.

Unless otherwise stated the melting points referred to are corrected.

PART I.

THE IN VITRO METABOLISM

OF

PREGNANE-3α:20α-DIOL.

1. Introduction.

The biochemistry of progesterone and pregnane-3α:20α-diol has been discussed in a number of excellent reviews of recent date (Pearlman (1948), Marrian (1949)). In this introduction therefore no attempt will be made to give a comprehensive survey of this field of investigation.

During the past decade the interest of investigators in the field of steroid biochemistry has turned from the more strictly chemical aspects of steroid isolation and structural determination to the problems of steroid hormone biosynthesis, the role of these substances in cellular processes, and their intermediary metabolism.

While the factual basis of our knowledge regarding the first and second of these problems is slight, much information has been gathered by different investigators on the changes which the steroid hormones undergo in the animal body.

In the case of the steroids with 21 carbon atoms, pregnane-3α:20α-diol, generally referred to as pregnanediol, has been established as a metabolic end product of the hormone progesterone in man (Venning and Browne(1937), Westphal and Buxton (1939)), and in the rabbit (Heard et al. (1941), Hoffman and Browne

(1942)). It is not however established that this is the only end product. Indeed pregnanediol is not found in the urine of certain species even after progesterone administration. Pregnanolones found in human pregnancy urine would appear to be likely metabolites of progesterone, but up to the present it has not been found possible to demonstrate their formation after the administration of progesterone.

The closely similar low values of urinary pregnanediol[x] excretion found after administration of either progesterone or pregnane-3α:20α-diol to human subjects suggest that although urinary pregnanediol[x] may be regarded as the excretion form of physiologically active precursors it may still be susceptible to further metabolic change (Sommerville and Marrian (1950)). Schneider and Mason (1948) express a similar view with regard to urinary ketosteroids

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x The term "pregnanediol" is used by the authors referred to here (Sommerville and Marrian (1950)) "to denote the material in urine consisting largely, but not necessarily entirely of pregnane-3α:20-diol which is determined by any one of the widely used quantitative procedures such as those of Venning (1937, 1938), Astwood and Jones (1941), Guterman (1944, 1945) and Sommerville et al. (1948)."

which may be regarded as excretion forms, having found that the urinary ketosteroid dehydroepiandrosterone yields Δ^5 androstene-3β:17α-diol and Δ^5 androstene-3β:16β:17α-triol on incubation with rabbit liver slices.

There has been much discussion regarding the sites of the metabolism of progesterone. Neither the ovary nor the uterus appear to be essential for the conversion of pregesterone to pregnanediol (Buxton and Westphal (1939), Buxton (1940), Sommerville and Marrian (1950)). It is well established that the liver of experimental animals is concerned in the metabolism of oestrogens and androgens (see reviews by Heard and Saffran (1949) and Samuels (1949)). Reports regarding the role of the liver in the metabolism of progesterone are however conflicting. Selye and Stone (1944) found that the anaesthetic action of orally administered progesterone was greatly increased by partial hepatectomy in rats. Dosne (1944) found less antifibromatogenic action when progesterone pellets were implanted in the spleen than when they were placed subcutaneously. Mussio-Fournier (1937-1940) found that progesterone injected into rabbit liver was less effective in producing progestational proliferation than when injected subcutaneously. Fels and Monaco (1941) could not confirm these results and expressed the view that the liver does not inactivate

progesterone. Kochakian (1944) compared the effect of pellets of progesterone implanted in various tissues, on the rabbit uterus, and concluded that the liver is the main organ concerned with progesterone inactivation. Masson and Hoffman (1945) found that progesterone administered to rabbits by stomach tube produced less progestational change in the uterus than when injected subcutaneously.

Zondek (1941) & Engel (1944) failed to obtain any inactivation of progesterone in vitro using liver pulp.

Indirect supporting evidence that the liver is involved in progesterone metabolism comes from the observations of Pearlman and Pincus (1946) that pregnane-3α:20α-diol was excreted in the bile of a post menopausal woman receiving Δ^5 pregnen-3β-ol-20-one, a possible precursor of progesterone, the discovery of pregnane-3β:20β-diol in ox bile (Pearlman (1946)) and the recent isolation of pregnane-3α-ol-20-one, pregnane-3α:20α-diol and aetiocholane-3α:17β-diol from the bile of pregnant cows. (Pearlman and Cerceo (1948)).

Summarising these observations it appears that in vivo the liver is an important site of the inactivation of progesterone. So far it has not been found possible to "inactivate" progesterone by liver in vitro.

The in vitro experiments reported by Zondek (1941) and Engel (1944) have involved the use of pulped liver and biological assay to determine "inactivation" of the progesterone. It appeared desirable to repeat these experiments using a suitable water soluble derivative of progesterone, intact liver cells, and some chemical method of determining change in progesterone itself or metabolic products formed.

The suitable water soluble derivatives and methods of chemical analysis were not immediately available, and it appeared that a simpler approach to the problem involved in this aspect of progesterone metabolism would be to investigate the in vitro action of liver on pregnane-3α:20α-diol. Chemical methods for determining pregnanediol exist and, as already pointed out, Sommerville and Marrian (1950) have shown that administration of pregnane-3α:20α-diol to human subjects leads to no greater excretion of urinary pregnanediol than the administration of an equivalent amount of progesterone, suggesting the possibility that progesterone may be more extensively reduced to pregnanediol than the amount excreted would indicate and that this substance may be largely metabolised further.

The dihemisuccinate sodium salt appeared

to be a suitable soluble derivative of pregnane-3α:20α-diol. Schneider and Mason (1948 (a) and (b)) have made successful use of hemisuccinates of dehydroepiandrosterone, androsterone and aetiocholan-3α-ol-17-one; Pearlman and De Mio (1949) have used an oestradiol hemisuccinate in in vitro experiments.

A quantitative method has been developed in the course of the present work for the extraction and determination of pregnanediol in tissue incubation mixtures.

This method has been used in an investigation of the destruction of pregnanediol on incubation with rat and rabbit tissues.

2. Materials and Methods.

(a) Tissue Preparations.

Unless otherwise stated the experimental animals were female rats of the Wistar Strain approximately 1 year old. They were killed by dislocation of vertebrae in the neck, and the required organs rapidly dissected out and placed in a small covered beaker standing in crushed ice.

Slices of liver about 0.3m.m. thick were prepared by the method of Deutsch (1936) as modified by Cohen (1945). They were not immersed in any solution but were placed when cut in a covered Petri dish standing on ice. A moist atmosphere was maintained in the dish by lining the bottom with hardened filter paper moistened with the calcium-free saline solution (pH 7.4) described by Krebs and Eggleston (1940). The filter paper was marked off in sectors corresponding to the number of samples required and, in order to ensure uniform sampling, slices were piled serially in the sectors. Samples were weighed on a torsion balance.

Brain slices were prepared in the same manner. In order to obtain sufficient tissue, slices from two or more brains were pooled.

Thin strips of muscle were prepared from rat adductor muscle as described by Field (1948).

The Liver acetone powder was prepared by homogenizing fresh liver with 10 times its volume of dry acetone, chilled in ice and salt, for 2-3 minutes in a well chilled Atomix Blender (Messrs. Measuring and Scientific Equipment, Ltd.). The homogenate was filtered through a chilled Buchner funnel taking care not to suck air through the dry filter. The filter cake was further washed twice with 5 times the volume of original liver of cold, dry acetone, twice with 5 volumes of cold, dry ether, in the Blender, and dried for an hour over P_2O_5 in a desiccator evacuated by oil pump. The powder was not stored, but was used immediately after preparation.

Both when handling fresh tissue and when preparing acetone powders, every effort was made to work as rapidly as possible at about 0°. In the case of liver and brain slices, with suitable technical assistance, experiments were started within 15-20 minutes of killing the animal. When preparing muscle strips, 45-50 minutes were required. The preparation of liver acetone powder from a single rat required 10 minutes between the death of the animal and the final ether wash.

(b) Preparation of Pregnanediol Dihemisuccinate. (PDHS)

Pregnanediol dihemisuccinate was prepared from 500 mg. weights of pregnane-3α:20α-diol m.p. 236-237° made in this Department from human pregnancy urine by Mr. I. Kyle.

500 mg. pregnanediol and 1.5 g. succinic anhydride were dissolved in 12.5 ml. dry redistilled pyridine and heated for 2 hours in a boiling water bath, taking precautions to exclude moisture. The solution which had darkened somewhat was then cooled, diluted with 200 ml. water containing about 50 g. crushed ice and extracted with 200 ml. followed by 4 x 100 ml. volumes ether. The combined ether extract was washed with four 100 ml. volumes 2N-H_2SO_4 and extracted with four 100 ml. volumes of 5% (w/v) Na_2CO_3. The carbonate extract was back extracted with 100 ml. ether, and carefully acidified to litmus with about 35 ml. 12 N-H_2SO_4. It was necessary to place the solution in a large flask to avoid loss from the very persistent frothing which occurred. The acidified solution was extracted with 200 ml. and three 100 ml. volumes ether. The ether was washed three times with 50 ml. volumes water, dried over $CaCl_2$ and evaporated.

In a typical experiment the yield at this stage was 480 mg.

The succinate was very soluble in cold methanol, ethanol, acetone, chloroform and benzene. It was rather more sparingly soluble in ether and almost insoluble in hexane. Crystallization was effected by dissolving the solid in a small volume of ethanol, adding hexane (about 10 volumes) until the hot solution became cloudy, boiling to clear and refrigerating.

The following results were obtained with a typical preparation - crystallized once from ethanol/hexane. After drying for 1 hour at 80° in vacuo over P_2O_5 the m.p. was 146.5-147.5°, found C, 67.4; H, 8.5%. Pregnanediol dihemisuccinate $C_{29}H_{44}O_8$ requires C, 67.2; H, 8.5%.

(c) Sodium Pregnanediol Glucuronidate.

The sodium pregnanediol glucuronidate used was prepared in this Department by Mr. Ian Kyle. The material was subjected to four treatments with Girard's Reagent T in order to remove ketonic material as described by Sutherland and Marrian (1947).

After drying at 137° for 10 hours in vacuo over P_2O_5 the m.p. was 274-276° (with decomposition). Sutherland and Marrian (1947) reported 283.5-284.5° (with decomposition).

Analyses of the dried material gave C, 62.6; H, 8.5; Na, 4.1%. Calc. for sodium pregnanediol glucuronidate $C_{27}H_{46}O_8Na$ C, 62.5; H, 8.4; Na 4.4%.

The toluene soluble hydrolysis product however did not give a pure "pregnanediol colour" in the H_2SO_4 reaction and the material cannot be regarded as entirely satisfactory.

(d) Preparation of Suspension Medium.

The suspension medium used in all experiments was the calcium free phosphate saline pH 7.4 of Krebs and Eggleston (1940). This will be referred to as the "saline solution".

The pregnanediol dihemisuccinate was dissolved in the saline solution in a concentration of about 0.595 mg./ml. by heating to about 75° for several minutes, filtering hot to remove any undissolved particles and cooling to 40°. Incubation flasks containing measured volumes of succinate solution were placed in the bath at 37° and removed momentarily to insert tissue preparations. In this way the maximum amount of steroid was maintained in solution. pH was adjusted to 7.4 when necessary after addition of any substance to the saline.

Saline solutions were saturated with oxygen or nitrogen as required. The nitrogen was not specially purified.

(e) Extraction and Determination of Pregnanediol.

A method was required for the quantitative extraction of pregnanediol from a suspension of tissue which had been incubated with P.D.H.S. solution and for the determination of pregnanediol in the extract.

In early experiments, tissue proteins in the incubated mixture were precipitated with 50 ml. dry acetone and filtered off. The precipitate was ground and washed with five 5 ml. volumes hot acetone. The combined acetone filtrate and washings were evaporated to about 10 ml. Any unchanged P.D.H.S. in this residue was saponified by heating under reflux 30 minutes with 8 ml. methanol and 2 ml. 25% (w/v) aqueous KOH. The methanol was then removed by evaporation under an air stream, 40 ml. water were added and the mixture was extracted with ethylene dichloride and subsequently with ether. Centrifuging was necessary to break emulsions formed with the first solvent. Extracts were washed separately with water and evaporated to dryness.

Attempts were then made to purify the pregnanediol in the combined residues by adsorption on alumina from dry ether and elution with wet ether. The pregnanediol recovered was determined by the

sulphuric acid colour reaction as described by Sommerville, Gough and Marrian (1948). The Spekker photoelectric absorptiometer readings were converted to weights of pregnane-3α:20α-diol by reference to a calibration curve made with known amounts of pure pregnane-3α:20α-diol varying from 0.1 to 0.5 mg.

Very variable results were obtained. The activity of the alumina and the dryness of the ether appeared to be very critical. The chromatographic technique was therefore replaced by the Astwood Jones precipitation method as modified by Sommerville et al. (1948). The residue from the ethylene dichloride and ether extracts was transferred quantitatively to a 20 ml. centrifuge tube and pregnanediol precipitated once with 0.1N-NaOH and once subsequently with water. A third precipitation was not found to be necessary. The second precipitate was stirred with 5 ml. ethanol and about 2 mg. "Norite" charcoal at 75°. The mixture was filtered through a No. 1 Whatman paper which had been well washed with hot ethanol (Verly et al. (1950)) into a 25 ml. graduated flask. The centrifuge tube and filter were washed with 3 x 2 ml. hot ethanol. After cooling, the filtrate and washings were diluted to 25 ml. with ethanol, and the solution thoroughly mixed.

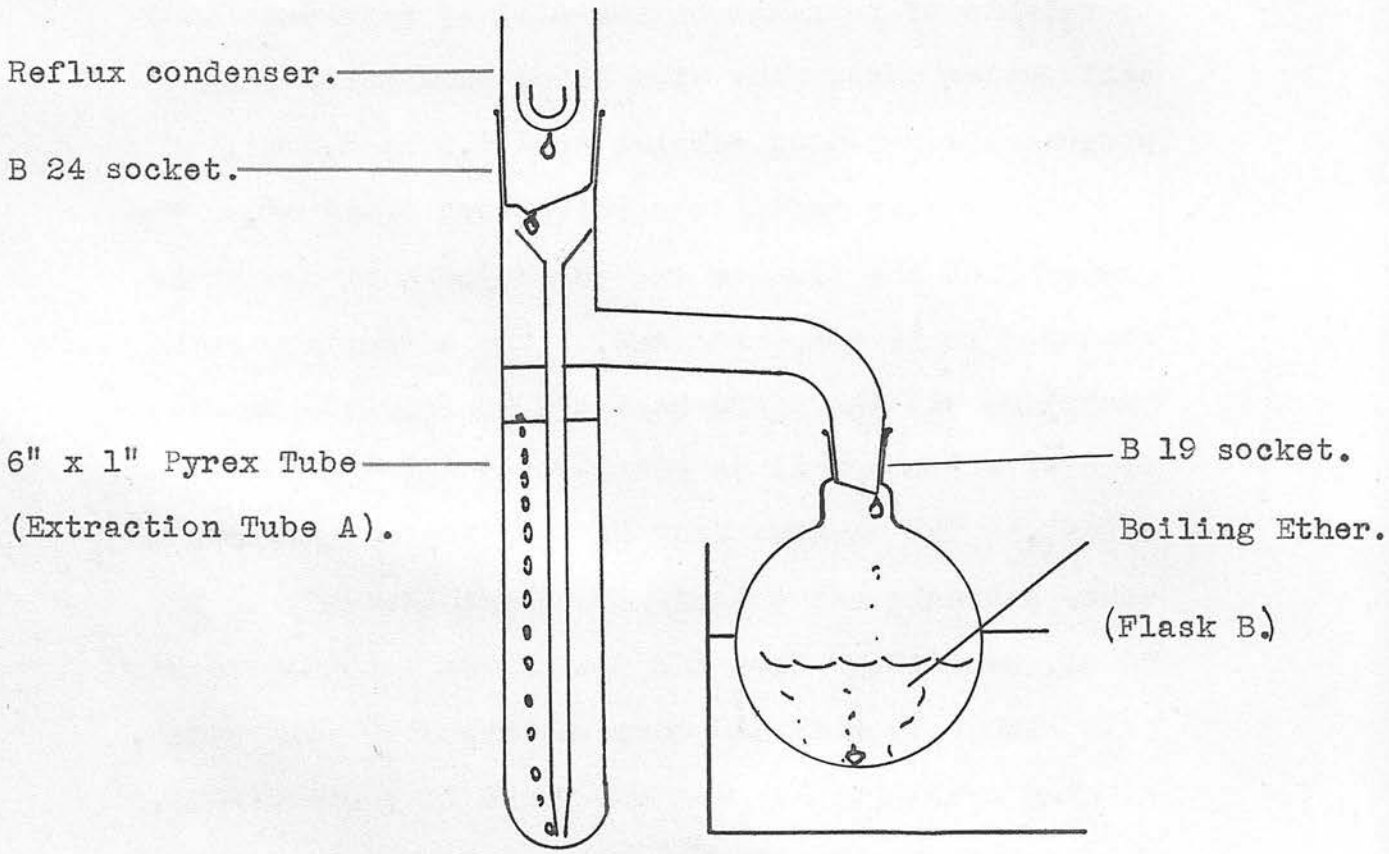


Fig. 1. Apparatus for continuous extraction of pregnanediol with ether.

5 ml. volumes were measured into 1 in. test tubes and evaporated under an air stream with the test tube in a hot water bath. Pregnanediol in the residue was determined by the sulphuric acid reaction already referred to.

Subsequently further modifications to the technique were introduced. In order to saponify any unchanged pregnanediol dihemisuccinate and to avoid possible loss due to adsorption of pregnanediol by the tissue preparations, at the end of the incubation period the contents of the flasks were diluted with 2.5 ml. saline solution and 5 ml. 10% (w/v) KOH solution. The flasks were closed lightly with glass bulbs and heated in a boiling water bath. In the case of controls, the incubated tissue suspension was poured into 2.5 ml. P.D.H.S. solution, which had been measured with the other volumes at the start of the experiment. The KOH solution was then added at once, and flasks were placed in the boiling water bath. After heating for an hour the tissue had dissolved. Flasks were removed from the bath. The stems of the bulbs were washed into the flasks with a few drops of ethanol, and contents of flasks were transferred to the extraction tubes A (See Fig. 1.) with three 2 ml. volumes of ethanol. The contents of the tubes

were mixed thoroughly and warmed in the water bath for a few minutes to assist solution of the pregnanediol. 8 ml. water were then added and the extraction apparatus was assembled with 50 ml. peroxide free ether in flask B. The ether was boiled sufficiently vigorously to ensure that a brisk stream of ether passed through the aqueous liquid.

Preliminary experiments indicated that more than 90% of pregnanediol could be recovered from saponified pure solutions of P.D.H.S. by 3 hours continuous extraction, adding 5 ml. ethanol to the aqueous phase at the end of the first and second hours.

At the end of the extraction period the ether was transferred to a separating funnel, washing the extraction flask into the funnel with 25 ml. ether. The ether extract was then washed twice with 15 ml. water and evaporated to dryness. The dry residue was transferred quantitatively to a 20 ml. conical centrifuge tube with ethanol and the determination of pregnanediol completed as already described.

With the adoption of these modifications the tissue blanks were satisfactorily low even with the high concentrations of cholesterol present in the brain experiments. The alkaline aqueous digest of the tissues extracted smoothly with ether without emulsion trouble except in a few cases in which the

stream of ether passing through the aqueous phase appeared to be too rapid. The work involved in the extraction and determination of the pregnanediol was considerably reduced by the adoption of continuous ether extraction. With a 2 hour incubation, 3 hour extraction, and overnight cooling of the first precipitate of pregnanediol, a complete experiment required slightly more than two working days.

(f) The Method Finally Adopted for the Determination of Pregnanediol in Suspensions of Tissue Preparations.

The steps in the method may be summarized briefly as follows:

1. Solution of incubated tissue and saponification of unchanged pregnanediol dihemisuccinate by heating for 1 hour at 100° with 5% KOH.

2. Transfer of alkaline solutions to extraction tubes, and three hours continuous extraction with ether.

3. Transfer of ether extracts to separating funnels, and washing of ether with water.

4. Evaporation of ether extracts and transfer of residues to centrifuge tubes.

5. Purification of pregnanediol present by the carefully controlled precipitation technique described

by Sommerville et al. (1948), omitting the third precipitation.

6. Solution of final precipitate in ethanol, treatment with charcoal and filtering.

7. Evaporation of accurately measured portions of the filtrate in 1" tubes.

8. Determination of pregnanediol in the residues by measuring the intensity of light absorbed by the colour developed with sulphuric acid and referring to a suitable "calibration curve".

(g) Accuracy of the Method.

The accuracy of the method was checked in a number of recovery experiments.

Recovery of pregnanediol after continuous extraction with ether for different periods.

250 mg. samples rat liver slices were incubated for 2 hours in 2.5 ml. saline and poured into 2.5 ml. volumes P.D.H.S. solution (equivalent to 957µg pregnanediol) in saline. 5 ml. 10% (w/v) KOH were added at once and the extraction and determination completed as already described. The time of continuous extraction with ether was varied.

The results are shown in the Table. 1.

TABLE 1.

Continuous Ether Extraction of Pregnanediol from Liver KOH digest.

Amount of pregnanediol added to liver KOH digest - 957 μg.

Extraction Time. hours.	Pregnanediol Recovered (after correction for blank). μ g.	% Recovery.
1. x	725.	76%.
2. xx	770.	81%.
3.	870.	91%.

x 5 ml. ethanol added to aqueous phase at end of first hour.

xx 5 ml. ethanol added to aqueous phase at end of first and second hours.

Recovery of Weighed Amounts of Pure Anhydrous Pregnanediol from Pure Saline Solutions.

Experiment 1. - 10.540 mg. pregnanediol which had been dried at 137° in vacuo over P_2O_5 were dissolved

in 10 ml. ethanol. 1 ml.(≡ 1.054 mg. pregnanediol) of this solution was added to 10 ml. 5% (w/v) KOH in saline. This mixture was not heated but was transferred to the extraction tube, extracted with ether and pregnanediol determined in the extract as already described. The amounts of pregnanediol recovered, corrected for solvent blank, were

1.055 mg. (100%).

1.065 mg. (101%).

Experiment 2. - Experiment 1 was repeated but in this case the mixture was heated for 1 hour at 100° after addition of KOH. The amounts of pregnanediol recovered, corrected for solvent blank, were

1.000 mg. (95%).

1.000 mg. (95%).

indicating some loss of pregnanediol on heating with KOH.

Experiment 3. - In this experiment 1.007 mg. pregnanediol in 1 ml. ethanol were added to 2.5 ml. saline solution. The ethanol was then largely removed by evaporation under an air stream when the pregnanediol appeared as small flocculent particles. The determination was then completed in the usual way after 1 hour heating with KOH. The amounts of pregnanediol

recovered, after correction for reagent blank, were

0.940 mg. (94%).

0.940 mg. (94%).

0.945 mg. (95%).

A 3 hour continuous ether extraction was employed in all experiments.

The pure solution blank in three experiments was equivalent to 0.030 mg. pregnanediol.

Conclusions.

1. A 3 hour continuous ether extract of KOH digest of incubated tissue recovers over 90% of added pregnanediol.

2. The recovery of weighed quantities of pure pregnanediol from pure saline solutions is satisfactory.

(h) Specificity of the Method.

The method described here for the determination of pregnanediol in tissue suspensions is no less specific than the method of Sommerville et al. (1948) for the determination of urinary pregnanediol. The only steroid present in considerable amounts in the tissues examined is cholesterol. The "tissue blanks" in experiments with brain slices will show that this steroid is effectively eliminated by the precipitation process employed.

Sommerville (1948) has reported on the behaviour of a number of steroids in the purification procedures and the sulphuric acid colour reaction. These steroids included allopregnane-3α:20α-diol, pregnane-3α-ol-20-one and its C_5 isomer. Sommerville concluded that the relative specificity of the method depends upon "a state of affairs whereby substances of high chromogenicity are eliminated, and substances which are not eliminated are weakly chromogenic" in the sulphuric acid reaction.

Progesterone would be destroyed by the heating with alkali in the extraction procedure adopted.

(i) Nature of the Blanks.

250 mg. tissue slices or liver acetone powder equivalent to 1 g. fresh weight liver were incubated with 2.5 ml. saline. The mixture was then subjected to the extraction, precipitations and sulphuric acid reactionsalready described. The Astwood Jones precipitations were found to be very effective in the removal of substances which might interfere with the sulphuric acid colour reaction, even in the case of brain tissue with its relatively high concentration of cholesterol.

"Solvent Blanks" were determined on solutions used without added tissue and without Astwood Jones precipitations. The results were not significantly different from the values found for "Tissue Blanks".

The following results are typical.

Blanks expressed as μg pregnanediol.

Saline only.	40, 45, 40, 33.
250 mg. liver slices in saline.	38, 45, 48, 35.
250 mg. brain slices in saline.	60, 25.
250 mg. muscle strips in saline.	45, 45.
Liver acetone powder = 1 g. fresh liver.	35, 35.

The blanks with liver acetone powder were low and very consistent.

3. The Arrangement of Quantitative Experiments.

Incubation of P.D.H.S. with Tissue Preparations.

250 mg. wet weight samples of liver or brain slices or muscle strips were placed in each flask, which contained approximately 1 mg. pregnanediol (as P.D.H.S.) in 2.5 ml. saline solution. In the case of acetone powders a weight of powder equivalent to 1 g. of fresh liver was used.

The dry weight of 250 mg. fresh liver slices was determined in a number of cases after drying samples to constant weight (2 hours) at 110°.

Inhibitors or methylene blue were added to the 2.5 ml. saline in 0.5 ml. volumes of saline after readjusting pH if necessary. In such experiments all volumes were usually adjusted to 3 ml. before incubation, either with inhibitor or pure saline solution.

After the various additions had been made to the incubation flasks they were gassed with oxygen or nitrogen as required for 30 seconds and closed with rubber stoppers. The flasks were then shaken continuously at a frequency of 90-100 per minute in a water bath at 37-38°. After 3 hours shaking, liver slices were largely intact. Brain slices were broken up.

Control Experiments.

In the controls, incubated suspensions of 250 mg. tissue in 2.5 ml. saline were added to 2.5 ml. P.D.H.S. saline solution followed immediately by the 5 ml. of 10% (w/v) KOH.

P.D.H.S. Analysis Samples.

All 2.5 ml. volumes of the fresh P.D.H.S. solution in saline were measured at the start of the experiment. Two 2.5 ml. volumes were reserved for pregnanediol analyses. They were subjected to 1 hour saponification with KOH, and 3 hours ether extraction as usual. The Astwood Jones precipitations were omitted. The dry residues from the ether extract were dissolved in ethanol, made up to 25 ml., and 5 ml. volumes were taken for the sulphuric acid reaction. Separate "solvent blanks" were determined and deducted from the weight of pregnanediol found only in the cases in which high tissue blanks were experienced. In other cases the "tissue blanks" were used. For liver and muscle they were not significantly different from the "solvent blanks".

Blanks.

In each experiment two samples of tissue were reserved for "tissue blank" determinations.

RESULTS.

(4) Destruction of Pregnanediol on Incubation with Rat Tissues.

In the following experiments samples of rat surviving tissue were incubated with P.D.H.S. in saline in an oxygen gas phase. Amounts of pregnanediol (p'diol) shown are corrected for blanks. Blank values are expressed as μg. pregnanediol.

(a) Incubation of P.D.H.S. with Rat Liver Slices.

	Blank.	μ g. pregnanediol in P.D.H.S. taken.	recovered after incubation.		recovered from controls.	
1.	28.	1062.	637.	60%.	952.	90%.
			502.	47%.	972.	93%.
2.	50.	1007.	590.	59%.	910.	90%.
			640.	64%.	940.	93%.
3.	30.	1085.	850.	78%.	975.	90%.
			780.	72%.	990.	91%.
4.	45.	997.	525.	53%.	895.	90%.
			545.	55%.	885.	89%.
		Means		61%.		91%.

Mean dry weights of 250 mg. fresh liver slices; Expt. 3 - 69 mg; Expt. 4 - 68 mg.

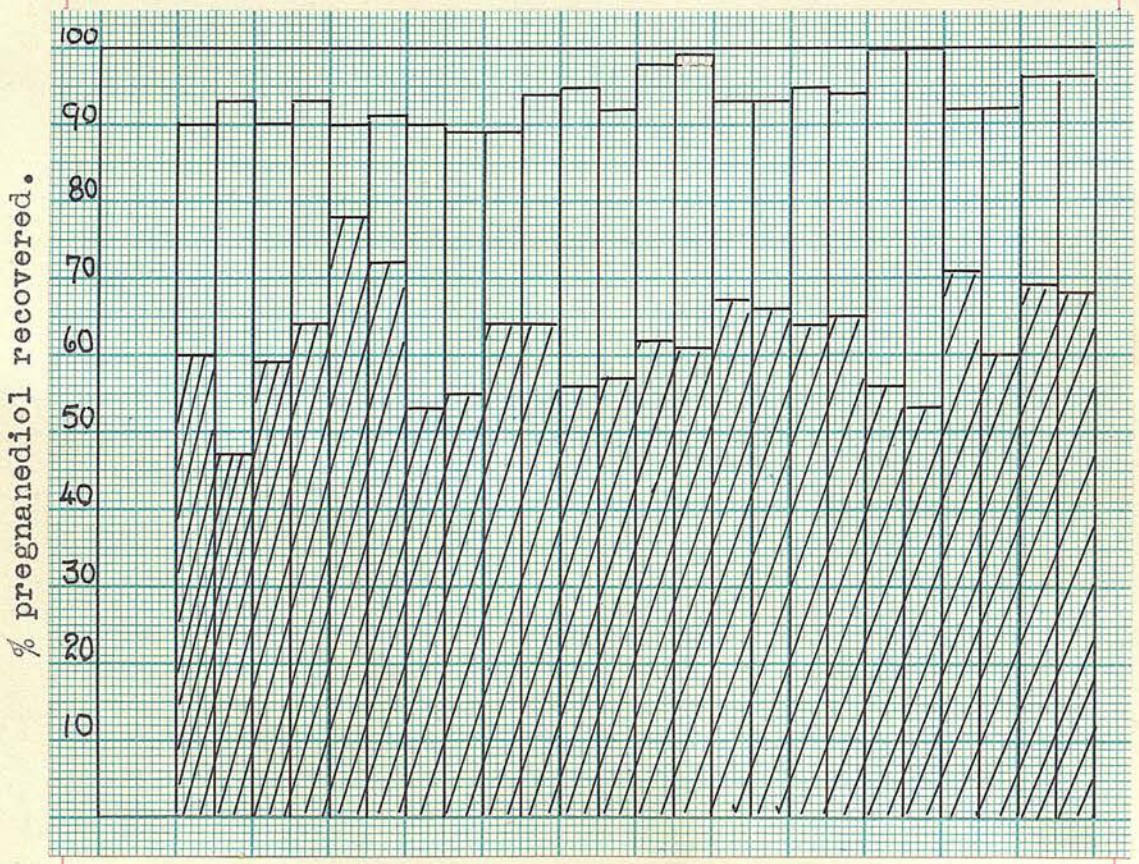


Fig. 2. Destruction of pregnanediol by rat liver slices. Cross hatched columns represent pregnanediol recovered after incubation. Full columns represent pregnanediol recovered from controls.

Experiment 1 belonged to an earlier series in which pregnanediol was extracted by shaking with ethylene dichloride rather then by continuous extraction with ether. In this experiment the incubation period was extended to 7.5 hours. In other experiments the period of incubation was 3 hours.

The results of experiments 1 to 4 and of all subsequent experiments in which liver slices were incubated with P.D.H.S. saline solutions in oxygen, e.g. as controls in inhibitor experiments are illustrated in Fig.2.

Conclusions.

Incubation of pregnanediol as P.D.H.S. with rat surviving liver slices in oxygen results in destruction of about 30% of the added pregnanediol. The destruction is not markedly increased by prolonged incubation, indicating that bacterial action is probably not involved.

(b) Incubation of P.D.H.S. with Rat Brain Slices.

μg. pregnanediol

Blank.	in P.D.H.S. taken.	recovered after incubation.	recovered from controls.
1. 60.	917.	780. 85%.	780. 85%.
		830. 91%.	805. 88%.
2. 25.	815.	750. 92%.	708. 87%.
		760. 93%.	720. 88%.

Conclusion.

Incubation of pregnanediol as P.D.H.S. with rat surviving brain slices in oxygen does not result in destruction of pregnanediol.

(c) Incubation of P.D.H.S. with Rat Muscle Strips.

μg pregnanediol.

Blank.	in P.D.H.S. taken.	recovered after incubation.	recovered from controls.
1. 33.	1008.	875. 87%.	920. 92%.
		925. 92%.	865. 86%.
2. 40.	827.	760. 92%.	735. 89%.
		725. 88%.	750. 91%.

Conclusion.

Incubation of pregnanediol as P.D.H.S. with rat surviving muscle strips in oxygen does not result in destruction of pregnanediol.

(d) Investigation of the Possibility that Disappearance of Pregnanediol on Incubation of P.D.H.S. with Liver is due to Conjugation.

The method of extraction of incubation mixtures would fail to recover alkali stable conjugated pregnanediol. Thus it seemed possible that conjugation might account for the disappearance of some of the pregnanediol. In order to investigate this possibility the extraction method was modified to include an acid hydrolysis. After the 1 hour heating with KOH the mixture was carefully neutralized with conc. HCl and an excess of 1 ml. of this acid was added. Flasks were then heated for 10 minutes in boiling water, cooled, made alkaline to litmus with KOH and transferred to the extraction apparatus. Blank experiments were performed in an identical fashion. Solutions of P.D.H.S. in saline for pregnanediol analyses were not subjected to acid treatment.

In one experiment the adequacy of the 10 minutes acid hydrolysis was checked by prolonging the heating for one hour. Although such treatment might be expected to result in some destruction of pregnanediol, destruction should be equally extensive in incubation experiments and controls. Any pregnanediol

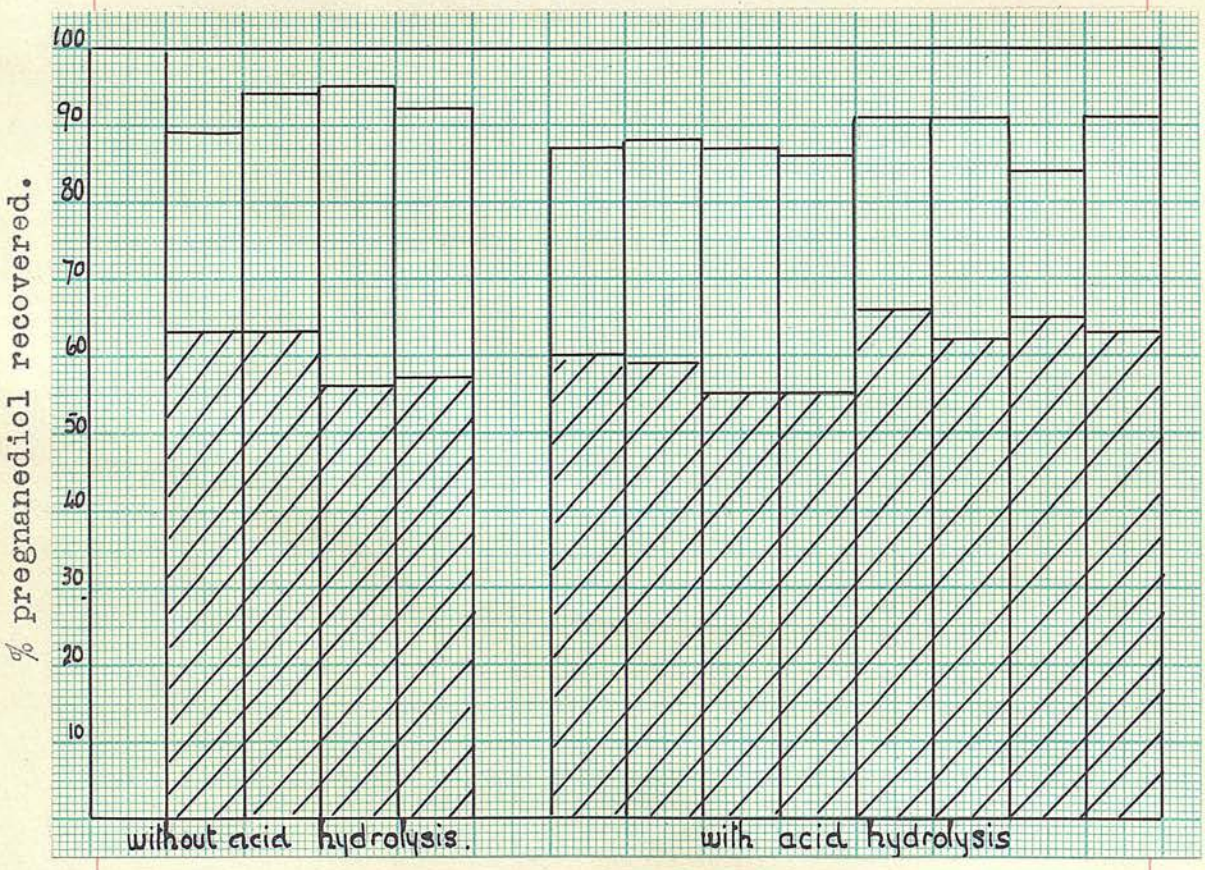


Fig. 3. Destruction of pregnanediol by rat liver slices. Cross hatched columns represent pregnanediol recovered after incubation. Full columns represent pregnanediol recovered from controls.

released from conjugation should increase the recovery of pregnanediol from incubated mixtures.

Incubation of P.D.H.S. with Rat Liver Slices with Subsequent Acid Hydrolysis.

μg. pregnanediol

Blank.	in P.D.H.S. taken.	recovered after incubation.		recovered from controls.	
		w/o acid hydrolysis.			
40.	952.	600.	63%.	850.	89%.
		595.	63%.	900.	94%.
		with acid hydrolysis for 10 mins.			
		570.	60%.	830.	87%.
		560.	59%.	840.	88%.
		w/o acid hydrolysis.			
30.	963.	535.	56%.	915.	95%.
		545.	57%.	890.	92%.
		with acid hydrolysis for 10 mins.			
		525.	55%.	840.	87%.
		525.	55%.	825.	86%.
		with acid hydrolysis for 1 hour.			
30.	955.	630.	66%.	865.	91%.
		595.	62%.	865.	91%.
		620.	65%.	805.	84%.
		600.	63%.	865.	91%.

See Fig. 3.

Conclusion.

Acid hydrolysis does not increase the amount of pregnanediol which can be recovered after incubation with liver. It therefore appears that the disappearance of pregnanediol is not simply a process of conjugation.

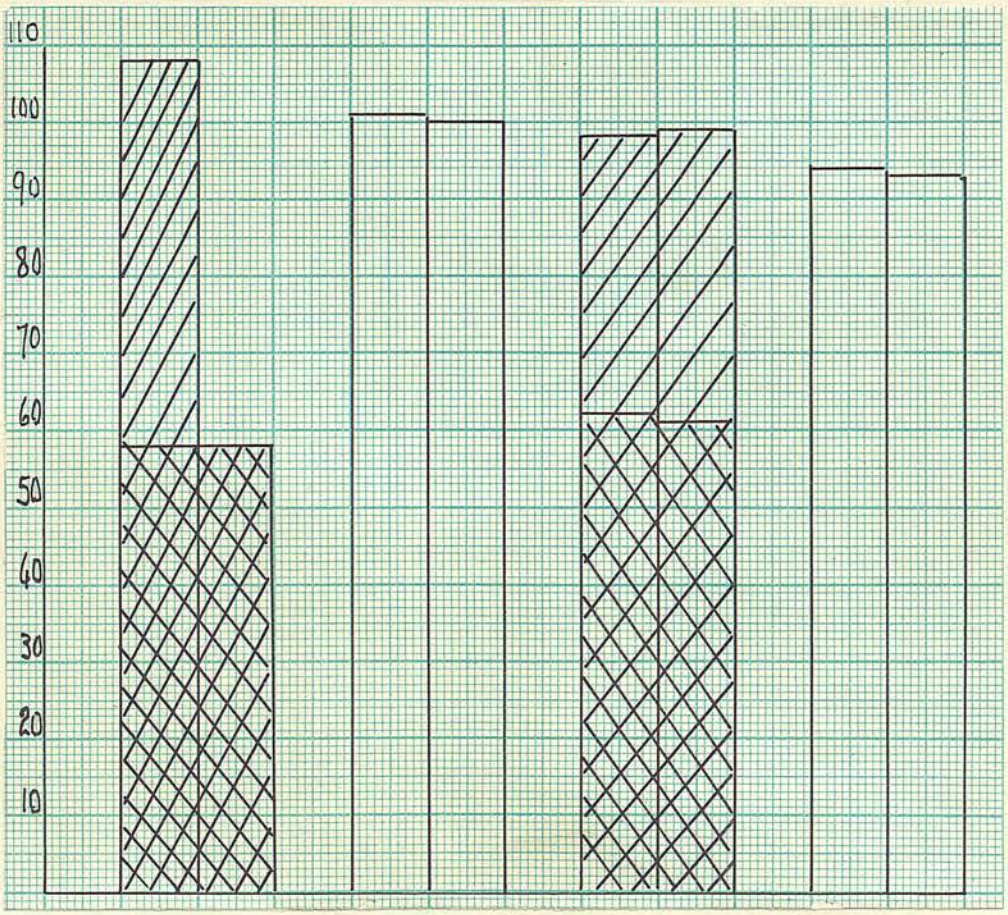


Fig. 4. Destruction of pregnanediol by rat liver slices inhibited in absence of oxygen.

% pregnanediol recovered from controls.

% pregnanediol recovered after incubation in oxygen.

% pregnanediol recovered after incubation in nitrogen.

5. Inhibition of Destruction of Pregnanediol by Liver.

(a) Experiments in Nitrogen.

In the following experiments samples of rat surviving liver slices were incubated with P.D.H.S. in saline in a nitrogen gas phase. Other samples were incubated in oxygen for comparison. "Controls" and "Blanks" were incubated in N_2.

	Blank.	μg. pregnanediol in P.D.H.S. taken.	μg. pregnanediol recovered after incubation.	μg. pregnanediol recovered from controls.
1.	30.	970.	In Oxygen. 560. 58%.	In Nitrogen. 1050. 108%.
			560. 58%.	
			In Nitrogen.	
			985. 101%.	
			975. 100%.	
			In Oxygen.	In Nitrogen.
2.	30.	1020.	660. 62%.	1000. 98%.
			650. 61%.	1005. 99%.
			In Nitrogen.	
			960. 94%.	
			950. 93%.	

Conclusion.

The destruction of pregnanediol on incubation of P.D.H.S. with liver slices is almost completely inhibited in absence of oxygen.

See Fig. 4.

(b) Influence of Azide.

In the following experiments samples of rat surviving liver slices were incubated with P.D.H.S. saline in oxygen without azide and in the presence of different concentrations of azide. The presence of azide made no difference in control experiments and blanks.

μg. pregnanediol

Blank.	in P.D.H.S. taken.	recovered after incubation.	recovered from controls.
0.001 M SOD. AZIDE.			
1. 35.	1080.	w/o azide.	
		720. 67%.	1000. 93%.
		715. 66%.	
		with azide.	
		795. 74%.	
0.01 M SOD. AZIDE.			
2. 50.	1055.	w/o azide.	
		680. 64%.	1005. 95%.
		690. 65%.	995. 94%.
		with azide.	
		815. 77%.	
		810. 77%.	

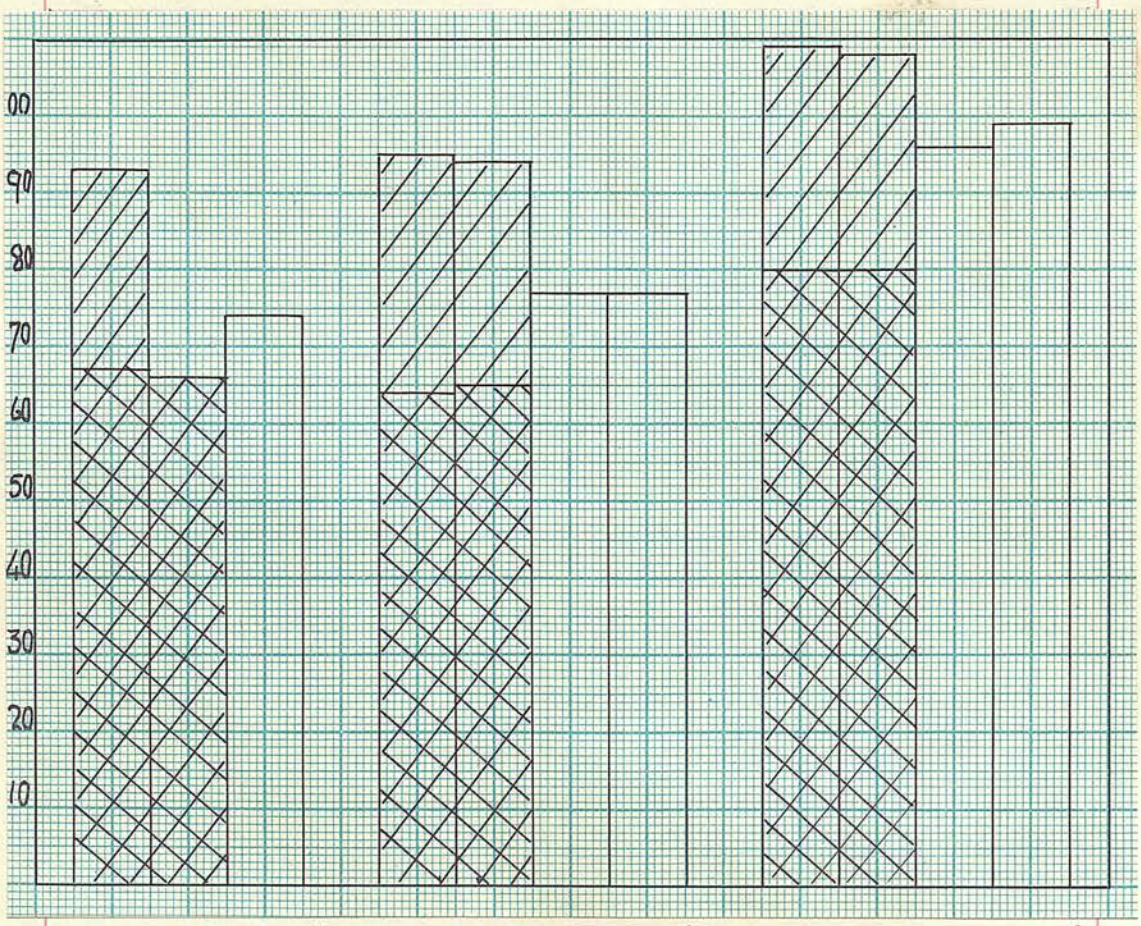


Fig. 5. Inhibition of the destruction of pregnanediol by azide.

▨ % pregnanediol recovered from controls.

▩ % pregnanediol recovered after incubation in absence of azide.

▭ % pregnanediol recovered after incubation in presence of azide.

μg. pregnanediol

Blank.	in P.D.H.S. taken.	recovered after incubation.	recovered from controls.
0.01 M SOD. AZIDE.			
3. 40.	1030.	w/o azide.	
		825. 80%.	1120. 109%.
		820. 80%.	1115. 108%.
		with azide.	
		985. 96%.	
		1020. 99%.	

Conclusion.

The destruction of pregnanediol on incubation of P.D.H.S. with liver slices is partly inhibited by sodium azide. 0.01M azide causes approximately 60% inhibition. See Fig. 5.

(c) Influence of Cyanide.

In the following experiments samples of rat surviving liver slices were incubated with P.D.H.S. saline in oxygen, without cyanide and in the presence of cyanide - 0.01M KCN.

μ g. pregnanediol

Blank.	in P.D.H.S. taken.	recovered after incubation.	recovered from controls.
1. 90. (35 "solvent blank")	1255.	w/o KCN.	
		705. 56%.	1250. 100%.
		670. 53%.	
		with KCN.	
		680. 54%.	
		715. 57%.	
2. 50. (35 "solvent blank")	1410.	w/o KCN.	
		995. 71%.	1290. 92%.
		845. 60%.	
		with KCN.	
		1055. 75%.	
3. 35.	1045.	w/o KCN.	
		720. 69%.	1000. 96%.
		715. 68%.	1000. 96%.
		with KCN.	
		735. 70%.	
		760. 72%.	

Conclusion.

The destruction of pregnanediol on incubation of P.D.H.S. with liver slices is not inhibited by 0.01M KCN.

6. Incubation of Sodium Pregnanediol Glucuronidate with Rat Liver Slices.

Sodium pregnanediol glucuronidate (NaPG) may be regarded as a more physiological water soluble substrate than the dihemisuccinate (P.D.H.S.) for these experiments. It was not however generally employed on account of the difficulty experienced in preparing pure specimens. Nevertheless it was felt that a few experiments with NaPG might yield interesting results. The specimen used (p. 10) was probably free from ketonic material but not of the highest purity.

14 mg. NaPG were dissolved in 24 ml. saline solution. 2.5 ml. volumes were used in the experiment in the same manner as the P.D.H.S. solution. In order to hydrolyse unchanged NaPG, incubated mixtures were heated for 1 hour with acid as described in Section 5 (d) (page 29.).

Samples of rat liver slices were incubated for 3 hours in an oxygen gas phase with NaPG in saline solution.

μ g. pregnanediol

Blank.	in NaPG taken.	recovered after incubation.	recovered from controls.
33.	635.	287. 45%. 327. 51%.	582. 92% 547. 86%.

Conclusion.

Incubation of NaPG with rat liver slices results in a disappearance of about 40% of the pregnanediol used.

7. Destruction of Pregnanediol by Liver Acetone Powder.

Liver acetone powders are more convenient than slices, since their use avoids the difficult operation of tissue slicing, and they give cleaner extracts after incubation. Experiments with acetone powders were undertaken with a view to their subsequent use in large scale isolation work, in which the cleaner extracts would be a distinct advantage. It was also hoped that more regular results would be obtained in the quantitative work by eliminating variations arising out of irregular slice thickness.

Preparation of the acetone powders has been described in Section 3 (a). A rat liver weighing about 10 g. gives approximately 2 g. acetone powder.

In the following experiments acetone powder equivalent to 1 g. fresh liver was used in each flask. The amounts of pregnanediol shown are corrected for blanks.

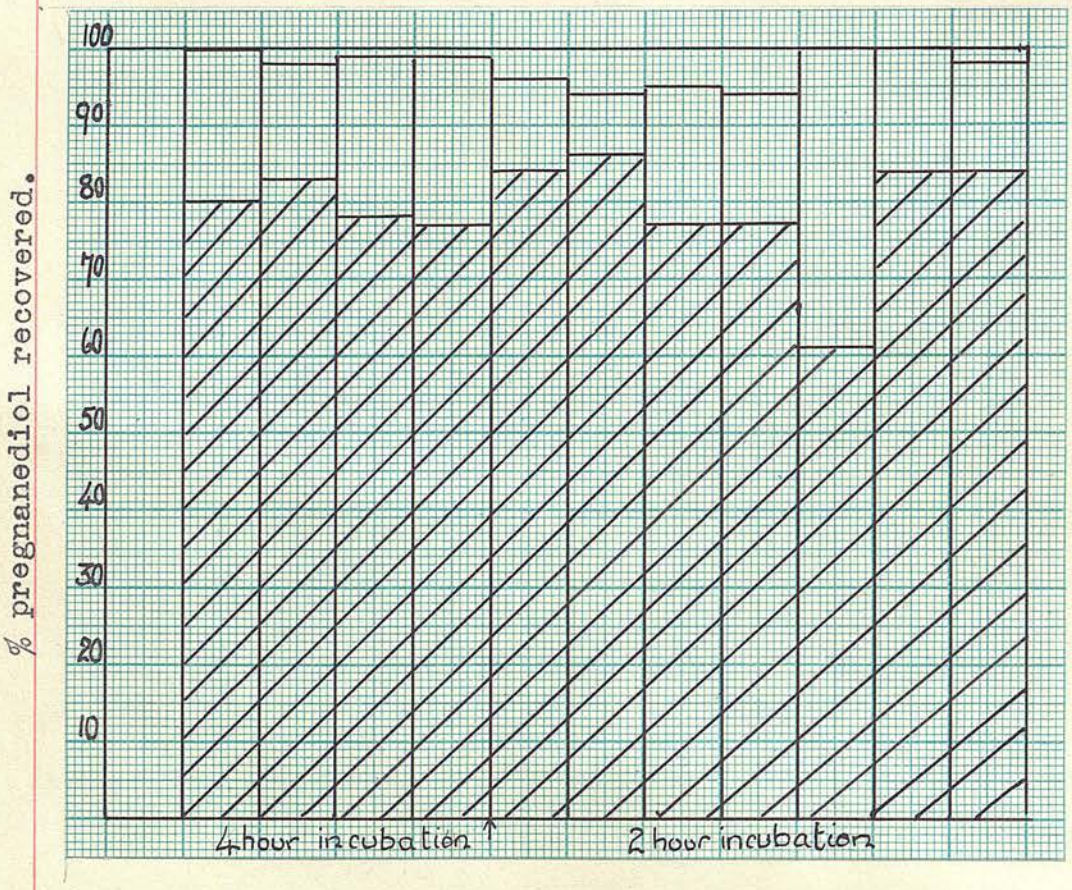


Fig. 6. Destruction of pregnanediol by rat liver acetone powder. Cross hatched columns represent pregnanediol recovered after incubation. Full columns represent pregnanediol recovered from controls.

(a) Incubation of P.D.H.S. with Rat Liver Acetone Powder.

Incubation for 4 hours in oxygen gas phase.

μg. pregnanediol

Blank.	in P.D.H.S. taken.	recovered after incubation.	recovered from controls.
35.	765.	615. 80%.	765. 100%.
		635. 83%.	750. 98%.

Conclusions.

Pregnanediol is destroyed on incubation of P.D.H.S. with rat liver acetone powder. The intact liver cell is apparently not necessary for this reaction. The activity of the acetone powder is however considerably less than that of the liver slices.

The results of incubating pregnanediol with rat liver acetone powders in oxygen are illustrated in Fig. 6.

(b) Destruction of Acetone Powder Activity by Heat.

Liver acetone powder samples equivalent to 1 g. fresh liver were added to P.D.H.S. saline solution at 100° and the mixture heated at 100° for 10 minutes. Subsequent incubations were in oxygen for 4 hours.

μg. pregnanediol

Blank.	in P.D.H.S. taken.	recovered after incubation.	recovered from controls.
35.	913.	<u>without heating</u>	
		715. 78%.	900. 99%.
		705. 77%.	900. 99%.
		<u>with heating at 100°.</u>	
		905. 99%.	
		910. 100%.	

<u>Conclusion.</u>

The ability of rat liver acetone powder to cause disappearance of pregnanediol on incubation with P.D.H.S. is destroyed by heat.

<u>(c) Experiment in Nitrogen.</u> 2 hours incubation.

μg. pregnanediol.

Blank.	in P.D.H.S. taken.	recovered after incubation.	recovered from controls.
35.	835.	<u>in oxygen</u>	
		700. 84%.	800. 96%.
		720. 86%.	780. 94%.
		<u>in nitrogen</u>	
		755. 91%.	
		795. 95%.	

<u>Conclusion.</u>

The disappearance of pregnanediol on incubation of P.D.H.S. with liver acetone powder is almost completely inhibited in the absence of oxygen.

(d) Experiments in Nitrogen in Presence of Methylene Blue.

These experiments were designed to investigate the possibility that the enzyme system responsible for the disappearance of pregnanediol on incubation with liver powders can use methylene blue as a hydrogen acceptor in lieu of atmospheric oxygen. A preliminary experiment with surviving liver slices indicated that a high concentration of the dye might be necessary.

2 hours incubation.

μg. pregnanediol.

Blank.	in P.D.H.S. taken.	recovered after incubation.	recovered from controls.
1.0mM methylene blue.			
35.	1213.	In oxygen.	
		945. 77%.	1050. 95%.
		945. 77%.	1030. 94%.
		In Nitrogen.	
		1150. 95%.	
		In Nitrogen + Me Blue.	
		1015. 84%.	
		1015. 84%.	

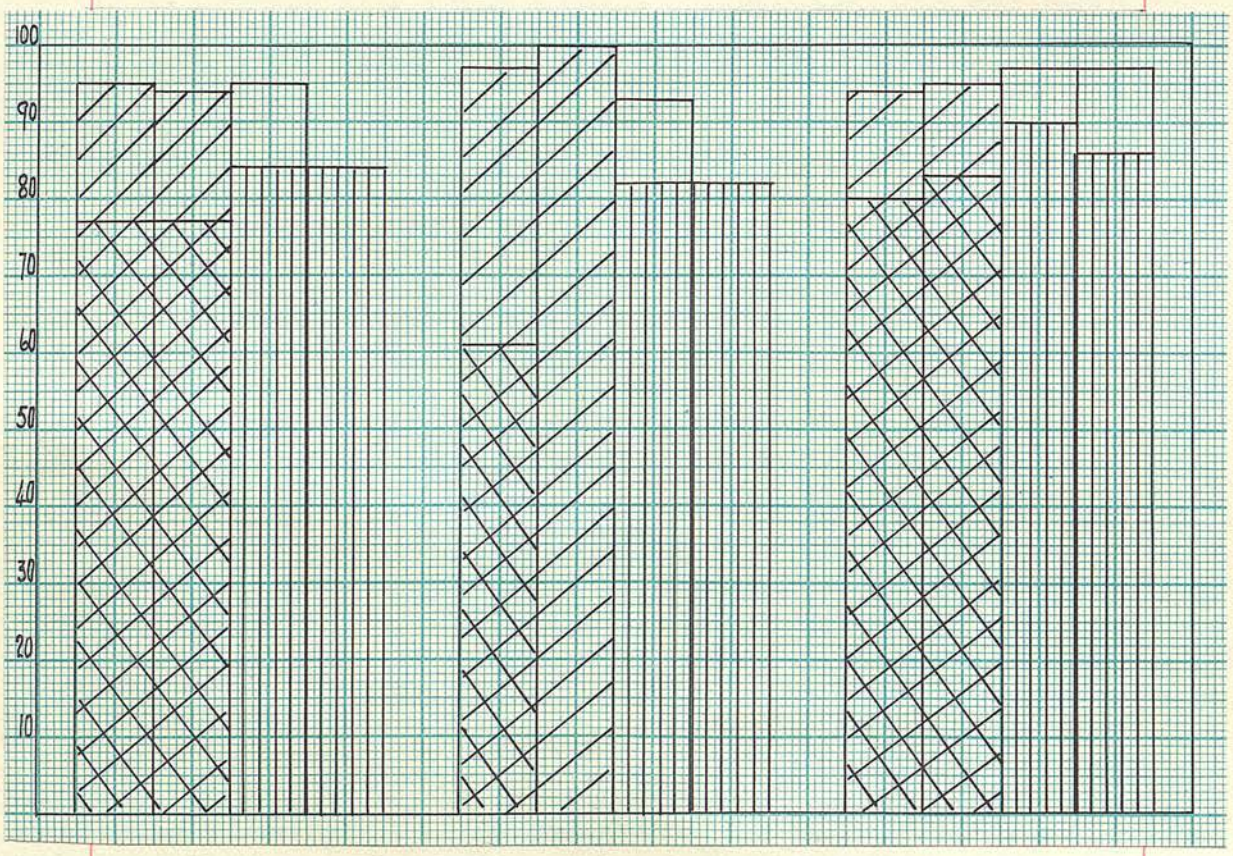


Fig. 7. Experiments in Nitrogen in Presence of Methylene Blue.

% pregnanediol recovered from controls.

% pregnanediol recovered after incubation in oxygen.

% pregnanediol recovered after incubation in nitrogen.

% pregnanediol recovered after incubation in nitrogen in presence of methylene blue.

μg. pregnanediol

Blank.	in P.D.H.S. taken.	recovered after incubation.	recovered from controls.
0.5mM methylene blue.			
35.	1035.	In oxygen.	1005. 97%
		630. 61%.	1035. 100%.
		In Nitrogen.	
		960. 93%.	
		In Nitrogen + Me Blue.	
		850. 82%.	
		850. 82%.	
35.	1040.	In oxygen.	
		835. 80%.	965. 94%.
		860. 83%.	985. 95%.
		In Nitrogen.	
		1005. 97%.	
		1005. 97%.	
		In Nitrogen + Me Blue.	
		930. 90%.	
		895. 86%.	

Conclusion.

Incubation of P.D.H.S. with rat liver acetone powder in a nitrogen gas phase results in the destruction of pregnanediol if methylene blue is added to the system.

See Fig. 7.

(e) Influence of Cyanide.

Rat liver acetone powder was incubated with P.D.H.S. in an oxygen gas phase for 2 hours, with and without added cyanide (0.01M KCN).

μg. pregnanediol

Blank.	in P.D.H.S. taken.	recovered after incubation.	recovered from controls.
35.	900.	without KCN.	
		760. 84%.	900. 100%.
		760. 84%.	885. 98%.
		with KCN.	
		735. 82%.	
		725. 81%.	

Conclusion.

The destruction of pregnanediol on incubation of P.D.H.S. with acetone liver powder is not inhibited by KCN.

8. Incubation of NaPG with Liver Acetone Powder.

It has been shown (Section 6, page 35) that incubation of rat liver slices with NaPG results in the destruction of pregnanediol. Since the pregnanediol C_{20} hydroxyl is free in NaPG it appeared that it might be possible to determine if this is a point of attack by liver enzymes. It seemed likely that protection of the C_3 hydroxyl by the glucuronic acid might be maintained during incubation with liver, since Karunairatnam and Levvy (1949) have shown that the hydrolysis of glucuronides by mouse liver β glucuronidase is 90% inhibited by 0.015M saccharate. Since it has not been clearly demonstrated that saccharate can enter the intact liver cell it was considered advisable to use liver acetone powder in lieu of slices.

In the following experiment a solution of 15 mg. NaPG in 24 ml. saline solution was used. 2.5 ml. volumes of this solution were incubated with rat liver acetone powder equivalent to 1 g. fresh liver for 2 hours in an oxygen gas phase. Potassium hydrogen saccharate in solution at pH 7.4 was added to two flasks to give a final concentration of 0.015M.

The extraction procedure involved a 10 minute acid hydrolysis of unchanged NaPG as described in section 5 (d), p. 28.

μg. pregnanediol

Blank.	in P.D.H.S. taken.	recovered after incubation.	recovered from controls.
35.	555.	w/o saccharate.	
		505. 90%.	570. 103%.
		515. 92%.	580. 105%.
		with saccharate.	
		580. 105%.	
		570. 103%.	

Conclusion.

No destruction of pregnanediol takes place on incubation of NaPG in the presence of saccharate under the conditions described above.

The results of this experiment are discussed in greater detail in the "General Conclusions" Section 11.

9. Experiment with Rabbit Liver Acetone Powder.

As it appeared likely that a considerable quantity of liver acetone powder would be required in attempts to isolate products formed on the incubation of pregnanediol with liver preparations, it was decided to investigate the activity of the livers of rabbits.

An acetone powder was prepared from the liver of an adult female rabbit. 30 g. liver gave 7 g. acetone powder.

Weights of powder equivalent to 1 g. fresh weight liver were incubated with P.D.H.S. solution in the usual manner for 4.5 hours in oxygen gas phase.

µg. pregnanediol.

Blank.	in P.D.H.S. taken.	recovered after incubation.	recovered from controls.
35.	1068.	790. 74%.	1085. 102%.
		800. 75%.	1050. 98%.

Conclusion.

Incubation of P.D.H.S. with rabbit liver acetone powder results in destruction of pregnanediol.

10. Preliminary Attempt to Isolate Products formed on Incubation of Pregnanediol as P.D.H.S. with Rabbit Liver Acetone Powder.

(a) Incubation Conditions.

P.D.H.S. was incubated with adult female rabbit liver acetone powder in six experiments. One animal was used for each experiment. The acetone powder from half of the liver was incubated with P.D.H.S. in saline solution in the ratio of powder equivalent to 1 g. liver to 1 mg. pregnanediol to 2.5 ml. saline. The acetone powder from the other half of the liver was incubated with saline solution as a control. The gas phase was oxygen. The incubation mixtures were divided into a number of flasks which were shaken continuously, to ensure good oxygenation, for 4 hours at 37.5°.

In this way acetone powder from 232 g. fresh liver was incubated with 232 mg. pregnanediol as P.D.H.S., and the same weight of liver powder was incubated in the control.

(b) Extraction of Incubated Mixtures.

After incubation the contents of flasks were homogenized in the Atomix Blender with 5 volumes

cold dry acetone and filtered. The filter cake was washed in the blender with half of the original volume of acetone and again filtered. The combined filtrates were evaporated to an aqueous residue of about 25 ml. This was diluted with 100 ml. water, acidified with H_2SO_4 and extracted once with 100 ml. and four times with 50 ml. volumes of ether. The combined ether extract was washed four times with 25 ml. volumes of 0.1N NaOH.

The combined alkali washings containing any unchanged hemisuccinate were acidified with H_2SO_4 and extracted with ether. The ether was washed with water, evaporated to dryness and the residue stored.

The alkali washed ether extract containing the "neutral fraction" was washed with 3 x 25 ml. volumes of water and evaporated to give a yellow waxy residue.

Controls were treated in the same way. The following weights were found for combined neutral fractions.

	P.D.H.S. Expts.	Controls.
(a) Expts. 1, 2 & 3. (114 mg. pregnanediol incubated).	325 mg.	257 mg.
(b) Expts. 4, 5 & 6. (118 mg. pregnanediol incubated).	245 mg.	134 mg.

(c) Separation of Ketonic Fractions.

Neutral fractions (a) and (b) were treated separately. Each fraction was dissolved in 10 ml. 95% (v/v) ethanol, 1.5 ml. glacial acetic acid and 0.5 g. Girard's Reagent T were added, and the mixture was refluxed for 1 hour. After cooling, the mixture was treated with 30 g. crushed ice and 11.5 ml. 2N-NaOH in a total volume of 88 ml. water. Non ketones were removed by extracting rapidly with 100 and 3 x 50 ml. volumes of ether. The combined ether extract was washed once with 25 ml. 5% (w/v) $NaHCO_3$, twice with 25 ml. water, evaporated to dryness and the non ketonic fraction stored.

The ketonic complex in the ether extracted aqueous phase was hydrolysed by adding 16 ml. conc. HCl and standing overnight. The ketones were recovered by extracting with 100 ml. and 3 x 50 ml. ether, washing the ether extract with 25 ml. 5% (w/v) $NaHCO_3$ and with 2 x 25 ml. water and evaporating to dryness.

The following weights were found for the ketonic fractions.

	"P.D.H.S. Expts."	Controls.
(a) Expts. 1 - 3. (114 mg. pregnanediol incubated).	8 mg.	2 mg.
(b) Expts. 4 - 6. (118 mg. pregnanediol incubated).	6 mg.	0.5 mg.

The fractions from the "P.D.H.S. experiments" were waxy films with some tendency to crystallize on "spotting" with hexane.

(d) Chromatography of Ketonic Fractions.

The combined ketonic fractions from the "P.D.H.S. experiments", dissolved in 15 ml. benzene, were poured onto a 10 x 5 mm. column of 0.5 g. Al_2O_3 (Peter Spence Type H; dried at 100° in vacuo; Brockmann and Schodder (1941) Activity II). The column was eluted successively with twenty two 5 ml. volumes of benzene, five 5 ml. volumes ether and ten 5 ml. volumes of acetone. The residues obtained on evaporation of the various eluates are described below.

Chromatography of Ketonic Fraction - "P.D.H.S. Experiment."

Benzene.		
1st. 15 ml. filtrate and 1st. 5 ml. eluate.	1.7 mg.	yellow gum.
2nd. to 22nd. eluates.	4.2 mg.	white crystalline films with traces of gum.
Ether.		
1st. to 5th. eluates.	1.6 mg.	white crystalline film + some clear gum.
Acetone.		
1st. to 10th. eluates.	2.7 mg.	white crystalline film + some yellow gum.

In an identical experiment the Control Ketonic Fraction gave the following results.

Benzene.

2 mg. yellow gum.

Ether.

- trace of gum.

Acetone.

- trace of gum.

No crystalline material could be obtained from the Benzene eluates.

(e) Further Treatment of Benzene Eluates from "P.D.H.S. Experiment" Chromatogram.

The 4.2 mg. partly crystalline material eluted with benzene in the "P.D.H.S. Experiment" were dissolved in 5 ml. warm hexane and subjected to a second chromatogram on 200 mg. of the same Al_2O_3. This was eluted with 9 x 2 ml.volumes of hexane giving 1.2 mg. gum and 0.5 mg. crystalline material, and with 12 x 2 ml. volumes of benzene giving 1.7 mg. crystalline material.

The material eluted with benzene melted at 143-145° on a Kofler type micro m.p. apparatus (Sutherland and Marrian (1947) reported 148-149.5° for authentic pregnane-3α-ol-20-one). An attempt

was made to purify this isolated material by sublimation at 100°/10^{-5} mm. Hg. The main sublimate was in the form of short needle shaped crystals which melted at 144.5-148°. Mixed with authentic pregnane-3α-ol-20-one (m.p. 146-148°) the m.p. was 144-147°.

It appears likely that the material isolated in very small yield from the benzene eluate of the chromatogram of ketonic fraction is pregnane-3α-ol-20-one.

(f) Acetone Eluate from "P.D.H.S. Expt." Chromatogram.

The partly crystalline material eluted by acetone was freed from traces of gum by chilling overnight in a few drops of acetone. The crystalline material melted at 232-235°. Mixed with authentic pregnane-3α:20α-diol (m.p. 235-237°) the m.p. was 233-236°. The isolated material eluted with acetone is therefore very probably pregnane-3α:20α-diol. Lack of material prevented a more thorough investigation of its identity.

Other Eluates.

Other eluates are being examined for ketonic oxidation products of pregnane-3α:20α-diol.

11. General Conclusions Regarding the Destruction of Pregnane-3α:20α-diol by Liver in Vitro.

It has been shown that rat and rabbit livers contain an enzyme system which is capable of destroying pregnane-3α:20α-diol. Failure to obtain marked increase in destruction when the incubation period was increased from 2 to 7 hours supports the view that the destruction is not due to bacteria. No increase in the recovery of pregnanediol was observed on acid hydrolysis after incubation, indicating that the disappearance of pregnanediol is not due to conjugation with glucuronic or sulphuric acids.

No destruction of pregnanediol was observed when rat surviving brain and muscle preparations were incubated with pregnanediol dihemisuccinate. It cannot be concluded however that these negative observations indicate the absence of the destroying enzyme system. These tissues may lack the esterases required to saponify the P.D.H.S. Experiments using NaPG as substrate have still to be carried out.

Heating the liver preparation for a short time at 100° prevents the destruction of pregnanediol during subsequent incubation. Replacing oxygen in the incubation flasks by nitrogen completely inhibits the destruction of pregnanediol. Azide causes partial

inhibition. No inhibition was observed in the presence of cyanide, indicating that the cytochrome system is not involved. The inhibition in nitrogen is partly released in the presence of methylene blue indicating that this dyestuff may replace atmospheric oxygen as a hydrogen acceptor for the enzyme system.

It seems possible that the enzyme in the liver responsible for the destruction of pregnanediol may be a dehydrogenase of the same type as xanthine oxidase or d-aminoacid oxidase, not requiring cytochrome/ cytochrome oxidase as the terminal oxidation system.

Since an oxidation process is involved it may be possible to increase the destruction of pregnanediol by addition of various substances which increase the oxygen consumption of tissues. Experiments along these lines are under consideration.

Using pregnanediol glucuronidate in a limited number of experiments, which cannot yet be regarded as complete, destruction of pregnanediol has been observed on incubation with rat liver preparations. Using acetone powder, and inhibiting β glucuronidase hydrolysis of NaPG by saccharate no destruction of pregnanediol was observed. This may indicate that a free C_3 hydroxyl is necessary for the destruction of pregnanediol. The saccharate may of course inhibit the enzyme system responsible for the destruction of

pregnanediol.

The unusually low percentage destruction of pregnanediol observed on incubating NaPG with liver acetone powder may be ascribed to the low activity of the β glucuronidase present at the pH of the incubation mixture (pH 7.4). The pH optimum for the hydrolysis of NaPG by β glucuronidase is likely to be about 4 - 5. In the experiment with NaPG and liver slices, the use of the intact cell renders the pH less critical and a greater destruction of pregnanediol was observed in this case. The relatively greater destruction of pregnanediol when using liver powders and P.D.H.S. may be due to the use of a pH (7.4) nearer the optimum of the esterase required to saponify the P.D.H.S. Attempts will be made to determine the pH optima of the esterase and pregnanediol enzyme. The fact that destruction of pregnanediol from the incompletely purified NaPG using liver slices was of the same order as that observed with P.D.H.S. under the same conditions would appear to discredit the explanation that impurities in the NaPG were responsible for inhibition of the enzyme in the later acetone powder experiments. The possibility that the cell wall offered a barrier to such inhibiting impurities must however still be considered.

The apparent failure of the enzyme system to attack unhydrolysed NaPG is disappointing. Hopes had been entertained that this water soluble pregnanediol derivative with a free C_{20} hydroxyl would prove a useful substrate for further study of the purified enzyme which causes destruction of pregnanediol. In such work the dihemisuccinate would be useless owing to the absence of esterases.

These experiments will be confirmed and extended when a purer sample of NaPG becomes available.

In one attempt to isolate products of the destruction of pregnanediol, after incubation of P.D.H.S. with rabbit liver acetone powder, pregnane-3α-ol-20-one was isolated in very small yield. Chromatography of the ketonic fraction isolated indicated that very small amounts of other ketonic substances may also have been present. It is possible that the pregnanediol which disappears on incubation with liver may be converted into a number of oxidation products. The possibility must also be considered that these small amounts of ketonic substances were contaminants in the sample of pregnanediol dihemisuccinate incubated. In view of the preliminary purification of the pregnanediol and preparation and purification of the P.D.H.S. this seems unlikely. Nevertheless it is intended to subject a second batch

of P.D.H.S. from the same source to the same processes, without tissue, and to look for ketonic contaminants.

The biochemical significance of this destruction of pregnanediol by the liver is not yet apparent. As already suggested the enzyme may be a dehydrogenase of the xanthine oxidase type or alternatively it may be a "reductase" which normally converts progesterone to pregnanediol acting in reverse. The relatively high concentration of pregnanediol added to the liver may stimulate this reversed reaction by a mass action effect. If the observation is confirmed it may be of significance that unhydrolysed NaPG is not attacked by the enzyme responsible for the destruction of free pregnanediol.

It is the author's intention to extend this work with a view to elucidating the biochemical significance of the destruction of pregnanediol which has been observed, and to investigate possible relationships between this destruction and the low recovery of administered pregnanediol, and the variations in the amounts of this substance excreted in various pathological conditions.

PART II.

THE CHEMISTRY OF OESTRIOL MONOGLUCURONIDIC ACID AND ITS SODIUM SALT.

1. Notes on Nomenclature.

By common use the term "oestriol glucuronide" or "oestriol monoglucuronide" is taken to mean the conjugated form of oestriol with glucuronic acid as found in pregnancy urine.

A more exact nomenclature uses the terms "oestriol monoglucuronidic acid" for the substance having a free carboxyl group, and "sodium oestriol monoglucuronidate" for the corresponding sodium salt. The abreviations HOG and NaOG will be used in this thesis for the free acid and sodium salt respectively.

The use of the less precise term "oestriol glucuronide" may be justified when referring to the conjugated substance as it exists in urine, as distinct from the isolated solid HOG or NaOG, since the glucuronide will be present in urine largely in the dissociated ionic form.

In accordance with the proposed nomenclature of Fieser and Fieser (1949) the term oestradiol-17β is used in this thesis to denote the more physiologically active 17 hydroxy epimer.

2. The Conjugated Forms of the Naturally Occurring Oestrogenic Hormones.

Little is known regarding the state in which the naturally occurring oestrogenic hormones, oestradiol-17β, oestrone, oestriol, equilin and equilenin exist in animal tissues and urine.

Marrian (1930 (a)) and Doisy et al (1930) independently observed that greater yields of oestrogenic substances could be obtained from human pregnancy urine, by extraction with immiscible fat solvents, if the urine was acidified before extraction. Marrian (1929 (a)) had already demonstrated the phenolic nature of the oestrogenic hormones in human pregnancy urine, and (Marrian 1930 (b)) gave a pK of about 10.5 for the substance later known as oestriol, the more acidic of the two oestrogens recognised at that time. Thus salt formation could not occur with these substances in normal fresh urine which is neutral or slightly acid, and the decomposition of salts could not be offered as an explanation of the increased yield of oestrogens which could be extracted from acidified urine.

Zondek (1934 (a)) claimed that the hormones could be completely extracted from urine without preliminary acidification but withdrew (Zondek 1934 (b)) in view of an investigation by Borchardt et al

(1934) of the optimum conditions for liberation of the hormones.

Butendandt (1931) suggested that the oestrogens in human pregnancy urine might be combined with a polyhydroxy organic acid. Later Marrian (1933) suggested that they might be conjugated with glucuronic or sulphuric acids.

Schachter and Marrian (1936) showed that at least part of the oestrogens in pregnant mares' urine occurred in conjugation with sulphuric acid, and two years later they succeeded in isolating oestrone sulphate from this source. The same substance was isolated from stallions' urine by Jensen et al in 1945. Butendandt and Hofstetter (1939) consider that oestrone is present in human pregnancy urine in conjugation with sulphuric acid. They approached the problem of the isolation of conjugated oestrogens by a study of the properties of oestrone sulphate which they synthesised. This work appears to have stopped when Schachter and Marrian reported their isolation of oestrone sulphate.

Using a modification of Kober's colour reaction for oestrogens (Kober 1931), Cohen and Marrian (1934 and 1935) made a study of factors influencing the hydrolysis of conjugated oestrogens in human pregnancy urine. Their method of chemical

assay, and information obtained on the stability of combined forms of oestrogens enabled them (Cohen and Marrian (1936)) to devise methods for the purification of these substances. This work culminated in the isolation of a combined form of oestriol from the urine of pregnant women which appeared to be an oestriol monoglucuronidic acid.

Subsequently Cohen, Marrian and Odell (1936) obtained this substance in the form of a crystalline sodium salt. The amounts of oestrone present in human pregnancy urine are so small that these investigators did not think it profitable to attempt to isolate the combined form.

It now appears that Collip's "emmenin" found in human placenta in 1930, and the substance of similar solubilities and physiological properties found in pregnancy urine (Collip 1932 (a), (b)) are probably oestriol monoglucuronide.

From the results of hydrolyses studies using an enzyme preparation thought to be a phenol sulphatase free from glucuronidase and esterases Cohen and Bates (1949) consider that varying proportions of the oestriol, oestrone and oestradiol present in human pregnancy urine may exist in conjugation with sulphuric acid.

Various authors have suggested that the naturally occurring oestrogens are present in the blood at least in part as protein complexes. (Rakoff et al (1943)). Szego and Roberts (1946) have suggested the existance of a β-globulin-oestriol glucuronide. They also make the interesting proposal that this may exist in equilibrium with a water soluble dialysable conjugate, presumably the oestriol glucuronide, suggesting a physico-chemical mechanism for the release of oestrogen at the cell membrane.

It is obvious that the nature and function of oestrogen protein complexes if they exist at all are still a matter for speculation. Satisfactory evidence regarding the nature of oestrogen conjugates is only available for these substances which have been isolated namely oestrone sulphate excreted by the stallion and pergnant mare, and oestriol glucuronide excreted by man. The latter substance has only been found in human pregnancy urine, and is the only known conjugate in man. A number of details of the chemical structure of this substance remained to be settled when the present work was taken up.

3. Previous Work on the Chemistry of Oestriol Monoglucuronidic Acid and its Sodium Salt.

In 1936 Cohen and Marrian isolated from human pregnancy urine an amorphous water-soluble substance containing about 50% of combined oestriol. The substance was also soluble in acetone, ethanol and ethyl acetate, and was insoluble in ether, benzene and chloroform. It could not be obtained in a crystalline form.

After drying in vacuo over $CaCl_2$ the substance melted at 193-197° (uncorr., decomposition) with sintering at 180°.

Elementary composition and barium content of the barium salt were in fair agreement with those required for an oestriol monoglucuronidic acid (HOG) ($C_{24}H_{32}O_9$). The analyses samples dried over P_2O_5 in vacuo lost 4.98% and 5.43%. The theoretical loss of weight for $C_{24}H_{32}O_9$ $1.5H_2O$ is 5.6%. The oestriol content was found by direct colorimetric measurement of the Kober colour to be 52, 53 and 49% (mean value 51%). The calculated value for $C_{24}H_{32}O_9$ $1.5H_2O$ is 56.6%.

Very shortly afterwards Cohen, Marrian and Odell (1936) reported the isolation of crystalline sodium oestriol monoglucuronidate (NaOG). When anhydrous methanol was used for the recrystallization

a product was obtained which melted with decomposition at 305-306° (uncorr.).

The results of analyses for carbon, hydrogen and sodium agreed well with a substance having the composition $C_{24}H_{31}O_9Na,0.5CH_3OH$. If the methanol used for recrystallization contained traces of water the product, after drying at 80°, in vacuo, over P_2O_5 melted at 256-257° (uncorr.) with decomposition, and gave analyses results in agreement with the structure $C_{24}H_{31}O_9Na,1.5H_2O$.

Two recrystallizations from anhydrous methanol raised the m.p. to 301-302°.

Marrian and his co-workers were unable at the time to obtain the solvent-free salt. The author has had the privilege of seeing the manuscript of a paper by Dr. S.L. Cohen, before publication, in which he now describes the preparation of the solvent-free sodium salt.

The optical rotation of the sodium salt (m.p. 305-306° uncorr.) was determined in a micro tube giving the following results:

$[\alpha]_D^{28°}$	C (water).
-28.2°.	0.4966.
-21.0°.	0.5027.

Opalescence of the aqueous solutions reduced the accuracy of the determinations.

Benedict's solution was not reduced until after hydrolysis, indicating that the oestriol is linked to the uronic acid by the terminal potential aldehyde group of the latter and not by an ester link. The formation of metallic salts containing one equivalent of metal provided further evidence in favour of this view.

The probable freedom of the phenolic hydroxyl in the complex was indicated by a strong positive Millon's Reaction in the cold. This was confirmed by Cohen, Marrian and Odell (1936), who observed that oestriol monoglucuronide in the presence of alkali showed the shift in absorption maximum from 280 to 295 mμ , which is characteristic of the naturally occurring oestrogens having a free phenolic hydroxyl. Further, they isolated an oestriol monomethyl ether from the products of hydrolysis of NaOG methylated with dimethyl sulphate. This methyl ether was identical with that obtained by methylating oestriol on the phenolic hydroxyl.

It is now clear that the uronic acid moiety is attached to the oestriol by a glycosidic linkage involving the potential aldehyde group of the former, and the C_{16} or C_{17} secondary hydroxyl group of the latter. The structure may be represented by

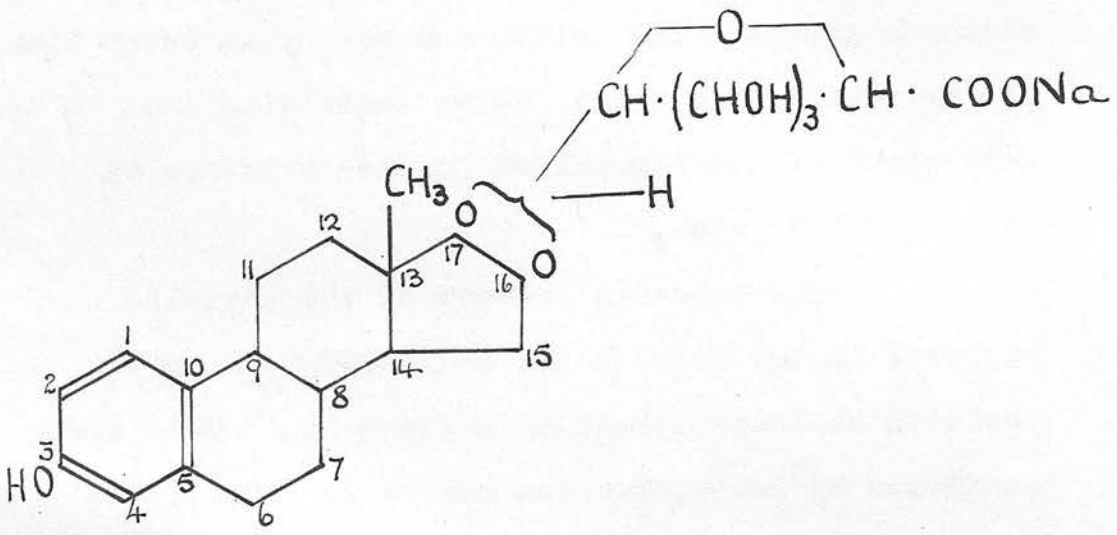


Fig. 8. Sodium Oestriol Monoglucuronidate.

the formula Fig. 8.

The unsatisfactory features of this structure will be discussed in the next section. No other work on the structure of NaOG has been recorded.

In this review of earlier work on oestriol glucuronide reference must be made to hydrolysis conditions. On account of the very great importance for satisfactory chemical assay of the urinary oestrogens many investigators have studied conditions of hydrolysis of the conjugated forms of these substances. The problem here is to effect complete hydrolysis of the ether insoluble conjugates with the minimum destruction of the ether soluble steroids liberated. This problem is not one of the chemistry of oestriol glucuronide alone, other oestrogens and other types of conjugation, for example probably with sulphuric acid, are involved. The subject has been reviewed by Van Bruggen (1948) and discussed recently by Heard and Saffran (1949).

4. Unsatisfactory Features of the Present Structure of NaOG.

No clear cut evidence has been obtained that the uronic acid moiety of NaOG is indeed glucuronic acid. In view of the lack of specificity of the Tollens reaction for glucuronic acid (Mandel and Neuberg (1908); Dische (1946)) it was felt that a further investigation of the uronic acid component should be undertaken.

Direct evidence regarding the anomeric form of the uronic acid in NaOG is lacking.

A pyranose type structure has been shown in Fig. 8 . There is at present no evidence which enables us to settle the ring size.

Finally, it is not known whether the uronic acid moiety is attached to C_{16} or C_{17} of the oestriol.

It was with the intention of settling some or all of these problems and of investigating the purity of NaOG prepared by procedures similar to those of Cohen, Marrian and Odell (1936) that the present work was undertaken.

5. The Preparation and Properties of Sodium Oestriol Glucuronidate (NaOG).

In their second paper on "oestriol glucuronide" Marrian and his co-workers (Cohen, Marrian and Odell (1936)) described an improved method for the isolation of this substance from human pregnancy urine as the crystalline sodium salt. The method takes advantage of the acidic nature of the complex and involves solvent partitioning between aqueous alkali and butanol and extraction by quinoline. The procedures were rather long but good yields as high as 500 mg. of fairly pure crystalline sodium salt were obtained from 30 l. urine.

The method employed in the present work is essentially similar. A few modifications including a continuous extraction of the urine with butanol have been introduced.

(a) Extraction of Urine.

The troublesome emulsions which frequently form when urine is shaken with butanol were avoided by adopting a modification of the procedure of Robinson and Warren(1948). These workers found that emulsions did not form with chloroform if it was injected in the form of a fine spray under the surface of the urine which was being extracted.

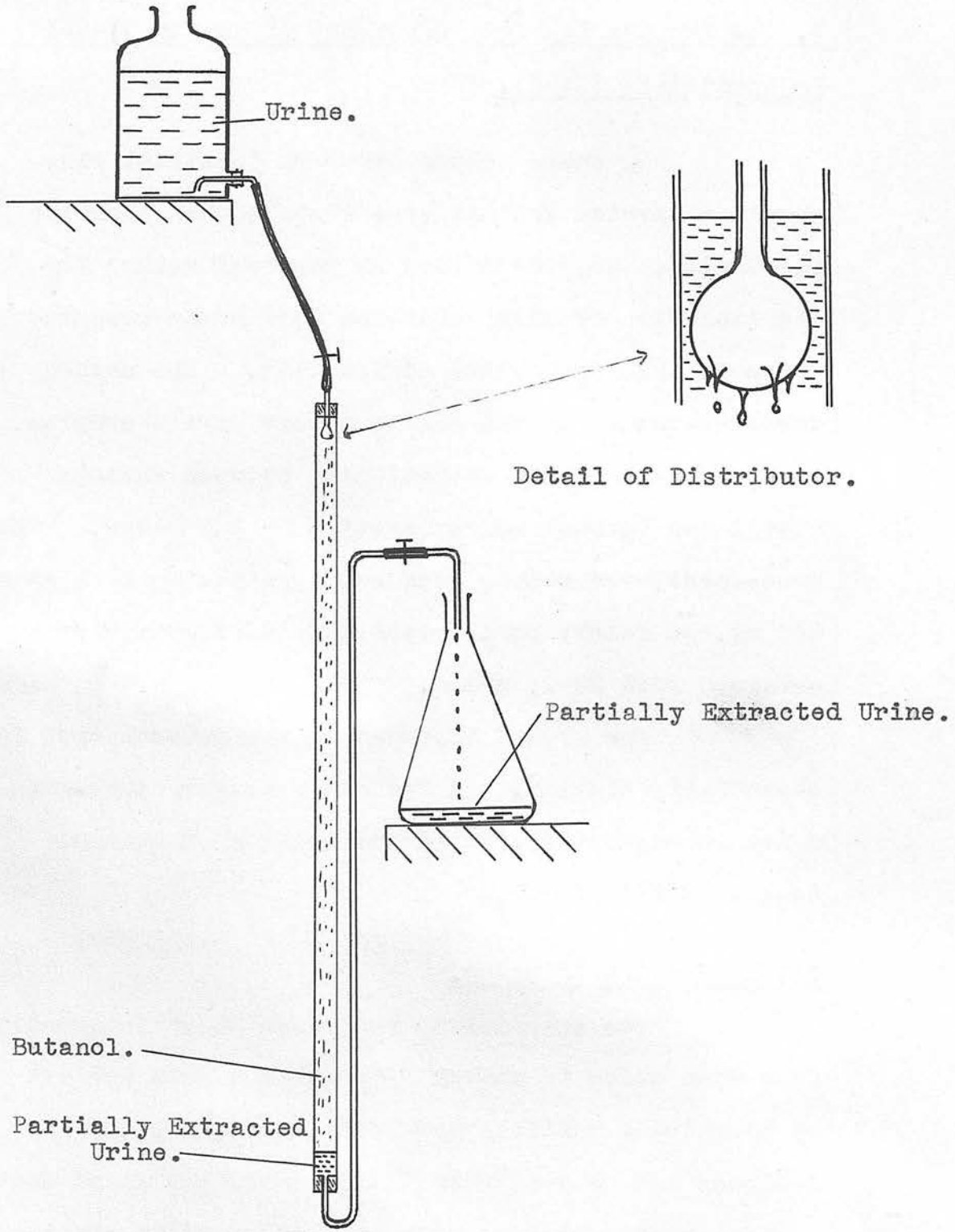


Fig. 9. Apparatus for the extraction of large volumes of urine

In the present work the urine was very conveniently extracted by spraying into columns of n-butanol. The n-butanol, purified by refluxing with NaOH and distillation, was contained in glass tubes 5 feet long and 1.1/2 inch diameter. The urine flowed into the columns, under gravity, through a number of fine capillaries opening under the surface of the butanol. No emulsions formed, and the pool of partially extracted urine collected at the bottom of the column was syphoned off continuously, and allowed to flow twice through a fresh column of butanol. In order to deal with larger batches of urine the columns were set up in batteries of three. After the extraction, butanol from the columns was pooled for further treatment.

The apparatus employed is illustrated in Fig. 9.

The urine was collected in batches of 5 to 10 l., acidified to pH 3.5 with HCl, and extracted with n-butanol as described. The pooled butanol extracts were neutralized with 10% (w/v) NaOH in butanol, concentrated under reduced pressure to one third volume, and extracted three times with 1/10 volumes 0.33N-NaOH. The combined NaOH extracts were stored in the refrigerator. When similar alkaline extracts from a total of 50 l. of urine had been

collected they were combined, acidified to pH 3.5, and extracted three times with 1/3 volumes of butanol. The combined butanol extracts were washed twice with 1/10 volumes water, made alkaline with 10% (w/v) Na_2CO_3 and evaporated to dryness under reduced pressure. The brown residue was dissolved in 500 ml. water, and the solution, after acidification to pH 3.5 was washed six times with 100 ml. volumes of benzene. The tar which separated and adhered to the walls of the glass-ware was dissolved in a minimum amount of warm acetone and added to the quinoline extract obtained later.

The benzene washed aqueous phase was then extracted six times with 100 ml. volumes of redistilled quinoline. The very dark brown quinoline phase separated rapidly on top of the lighter coloured aqueous phase. The quinoline was washed four times with 100 ml. volumes of 10% (w/v) Na_2CO_3 solution. Separation of the two phases in this case was very slow. The combined carbonate washings were back extracted once with 100 ml. quinoline. The combined quinoline extract was fairly rapidly extracted with six 100 ml. volumes of 0.1N-NaOH. The NaOH extracts were combined, acidified to pH 3 with HCl and extracted six times with 100 ml. volumes of butanol. The butanol extract was washed twice with 50 ml. volumes of water and evaporated to dryness under reduced pressure.

(b) The Separation of crude NaOG.

The dark brown gummy residue from the butanol extract was dissolved in about 50 ml. absolute methanol and the pH of the solution adjusted to about 7 by the addition of methanolic NaOH. This adjustment of pH is not easy, the most satisfactory results were obtained using Johnson Universal Comparator Test Papers moistened with water to indicate the required pH. The methanolic solution was slowly evaporated to about 25 ml. under a stream of air with the flask standing on a warm sand bath. The water bath was not satisfactory; possibly the methanol adsorbed water vapour rapidly and the separation of NaOG was thus prevented. A light brown precipitate separated from the methanol. After chilling, this was filtered off and washed with cold methanol. The yields of this light brown solid from 50 l. batches of urine varied greatly. On the average 10 mg. per litre of urine were obtained. Occasionally no solid could be obtained at all although a quantitative Kober Test on a small portion of the dark brown methanolic solution would indicate the presence of considerable amounts of oestrogen. On other occasions the solid separated if the contents of the flask were diluted with more methanol and then allowed to stand in the laboratory for a few days.

(c) Purification of crude NaOG.

The crude product was purified in the following way: repeated leaching of the solid with hot moist butanol dissolved the sodium oestriol 'monoglucuronidate' leaving a small amount of undissolved dark coloured tar. The combined butanol leachings were extracted three times with 1/10 volumes of 0.33N-NaOH; the combined alkaline extracts were acidified to pH 3.5 with HCl, and extracted three times with third volumes of butanol. The butanol extract was washed twice with 1/10 volumes of water, and taken to dryness under reduced pressure. The residue was dissolved in about 500 ml. methanol, made just alkaline with a methanolic solution of NaOH, boiled with a small amount of 'Norit' charcoal, and filtered while hot. The filtrate was concentrated to about 1/5 volume. During the concentration sodium oestriol 'monoglucuronidate' separated as a nearly white crystalline solid. After chilling, the solid was filtered, washed with a little cold methanol, and dried in vacuo.

This material did not give satisfactory C and H analyses. Recrystallization from ethanol containing 10% (v/v) water gave beautiful fine white needles but yields were poor.

It was found to be more satisfactory to

convert the sodium salt to the free glucuronidic acid, without previous crystallization, by the method to be described in a subsequent section (page 73). Pure NaOG was then obtained from the pure HOG by dissolving this in anhydrous methanol, adding an equivalent of NaOH in methanol and evaporating the solution to a small volume under an air stream. The white crystalline NaOG which separated was filtered off after chilling, and washed with cold dry methanol.

(d) Properties of Purified NaOG.

A batch of almost 1 g. pure NaOG was prepared from purified HOG (Section 6) as described in the previous paragraph.

A sample after drying over P_2O_5 at 137° in vacuo for 8 hours melted at 245-248.5° with decomposition.

C and H analyses of the dried sample gave mean values C, 59.6; H, 6.5; Na 4.6%. Calc. for sodium oestriol monoglucuronidate $C_{24}H_{31}O_9Na$ C, 59.3, H, 6.4; Na 4.7%.

The dry salt was found to be very hygroscopic and proved troublesome to handle on the micro balance. Thus after drying to constant weight over P_2O_5 at 137° in vacuo, a sample picked up 17.1% moisture on equilibration with the laboratory atmosphere.

As this particular batch of NaOG, prepared from purified HOG, appeared to be purified as thoroughly as existing methods permit it was used for the experiments designed to identify the uronic acid moiety. (Section 7 page 80).

Other preparations of NaOG which did not give satisfactory analyses results, even after purification by way of the free acid HOG, melted at higher temperatures. Cohen, Marrian and Odell (1936) gave an uncorrected m.p. of 305° for material analysing for NaOG with 0.5 molecules of methanol of crystallization and 256° for NaOG with 1.5 molecules of water. These "melts" take place with decomposition. The sudden frothing of the darkened material in the capillary is taken as the "melting point". The author considers that such m.p. are of very little value for the characterisation of these substances. The exact thermometer reading depends to a large extent on the rate of heating. No improvement was obtained on carrying out the m.p. determinations in capillaries evacuated by oil pump.

6. The Preparation and Properties of Oestriol Monoglucuronidic Acid. (HOG).

(a) The nearly pure crystalline NaOG which had separated from methanolic solution as described in Section 5(c) page 70 , was dissolved in a minimum of butanol saturated with water at about 60°. The solution was adjusted to pH 1 with HCl, washed with 1/10 volumes water till the washings were free from chloride, and taken to dryness under reduced pressure. The residue was dissolved in a minimum of boiling ethanol and precipitated from solution with 10 volumes of cold dry ether. The white amorphous solid oestriol 'monoglucuronidic' acid was filtered off and dried in vacuo. The product was successfully freed from solvents by repeated wetting with warm water, drying over $CaCl_2$ and subsequently over P_2O_5 in vacuo at 80°.

It was not found possible to obtain the acid in crystalline form. Slow evaporation of aqueous ethanolic solutions resulted in the formation of opalescent gels. These dried in the vacuum desiccator to amorphous white powders.

(b) Melting Point Data.

The following results were obtained with a satisfactory preparation of approximately 1 g. of HOG. After wetting with warm water and drying to

constant weight in vacuo at 80° over P_2O_5 the melting point was 224-226° with decomposition. Cohen, Marrian and Odell (1936) found that different preparations had sharp melting points ranging from 196° to 236°, and ascribed thedifferent melting points to varying amounts of water associated with the molecules of the acid.

(c) Specific Rotation.

The specific rotation was determined in ethanol using a Fischer Micro Tube. The sample was dried to constant weight at 80° in vacuo before weighing.

$$[\alpha]_D^{17^\circ} = -7.5^\circ \pm 0.8 \qquad \text{(C, 1.061).}$$

The specific rotation of HOG has not been recorded before.

(d) Analyses.

The mean values found in analyses of a sample dried to constant weight in vacuo at 80° were

C, 62.2; H, 6.9%.

Calculated for oestriol monoglucuronidic acid $C_{24}H_{32}O_9$ C, 62.0; H, 7.0%.

(e) Equivalent of HOG.

The equivalent was determined by dissolving accurately weighed quantities of HOG dried for 10 hours at 80° in vacuo over P_2O_5, in ethanol containing

20% (v/v) water and diluting to 10 ml. 1 ml. volumes were taken for titration with standard KOH solution using a Conway Burette. Thymol Blue was used as indicator.

1. 5.208 mg. HOG dissolved in 10 ml. solution.
   1 ml. required 0.220 ml. 0.00508 N-KOH.
   Hence HOG solution is 0.001118 N.
   equivalent is 0.5208/0.001118 = 458.
2. 5.30 mg. HOG dissolved in 10 ml. solution.
   1 ml. required 0.202 ml. 0.00571 N-KOH.
   Hence HOG solution is 0.001153 N.
   equivalent is 0.530/0.001153 = 460.

Theoretical value of equivalent = 464.5.

(f) Solubility of Oestriol Glucuronidic Acid in Water.

The solubility in water was determined approximately as follows. Samples of 12 mg. HOG were dissolved in 3 ml. water at 100° in 10 ml. centrifuge tubes. The tubes were cooled to 37° for 2 hours in the incubator, then centrifuged for 2 hours. The temperature of the solution after centrifuging was 27°. Undissolved HOG had separated as a gelatinous plug at the bottom of the tube. 1 ml. of the supernatant solution at 27° was pipetted onto a watch glass

and dried to constant weight in vacuo over P_2O_5.

The values for solubility at 27° found in three determinations were:

1.9 mg./ml.

1.7 mg./ml. Mean 1.7 mg./ml.

1.5 mg./ml.

(g) Distribution Coefficient of HOG between Ether and Water and between Ether and N-HCl Solution.

4.062 mg. anhydrous HOG were dissolved in 100 ml. water and shaken at frequent intervals during one hour with 100 ml. peroxide free ether saturated with water. The stoppered graduated cylinder containing the solutions was immersed in a thermostat at 18°. At the end of the hour the volume of the ether phase was 89 ml. and that of the water 110 ml. The oestriol glucuronide in 30 ml. portions of the ether phase was determined by the quantitative Kober reaction using the technique of Stephenson and Marrian (1947). The photo electric absorptiometer (Spekker) readings obtained were referred to a calibration curve prepared with known weights of pure HOG. The ether phase of 89 ml. was thus found to contain 0.159 mg. HOG. The molar concentration of HOG in the ether

phase is therefore

$$\frac{0.159 \times 1000}{464.5 \times 89} = 0.00394$$

the molecular weight of HOG being 464.5.
The molar concentration of HOG in the aqueous phase was similarly

$$\frac{(4.062 - 0.159) \times 1000}{464.5 \times 110} = 0.0765.$$

Thus the distribution coefficient of HOG between ether and water at 18° is

ether/water = 0.00394/0.0765 = 0.0515.

The determination was repeated using N-HCl instead of water, in order to suppress the ionization of the HOG. The distribution coefficient of HOG between ether and N-HClat 18° is

ether/N-HCl =0.0872/1.

Ether therefore would not be a satisfactory solvent for the extraction of HOG from pure aqueous solution.

(h) Water Absorption Experiment.

A sample of HOG dried to constant weight over P_2O_5 at 80° in vacuo weighed 97.7 mg. This was exposed to air saturated with moisture at 18°.

After 98 hours the weight became constant at 125.8 mg. representing an uptake of 28.1 mg. or 28.7% moisture. On drying over anhydrous $CaCl_2$ at atmospheric temperature and pressure the sample attained equilibrium after 49 hours losing 24.0 mg. Subsequent drying over P_2O_5 at 80° in vacuo resulted in a further loss of 4.5 mg.

TABLE 2.

Water Absorbed and Lost by HOG.

	Weights of Sample. (mg.)	Moisture Content. %	Moles of Water.
Anhydrous material.	97.7		
After exposure to moist air at 18° for 98 hours.	125.8	28.7	7.4.
After drying over $CaCl_2$ at room temp. and pressure for 49 hours.	101.8	4.2	1.95.
After drying over P_2O_5 at 80° in vacuo.	97.3		

Cohen and Marrian (1936) reported that samples of their HOG preparation dried over P_2O_5 at 80° in vacuo lost 4.95% and 5.43% of their weights. The theoretical loss for $C_{24}H_{32}O_9$, $1.5H_2O$ = 5.6%.

Considered along with these observations, the results of the experiment now reported would appear to indicate that although HOG is somewhat hygroscopic no definite hydrates are formed.

7. Identification of the Uronic Acid from Oestriol "Monoglucuronidic" Acid.

The identification of uronic acids is difficult, and the choice of method was restricted by the small quantity of purified oestriol "monoglucuronidic" acid which could be prepared. Unequivocal methods such as the isolation of D-glucurone as used by Pryde and Williams (1933) in the case of borneol glucuronide, or the preparation of the p-toluidine complex of ammonium glucuronidate (Smith and Williams (1949)) are not recommended for work with small quantities of glucuronide (Williams (1949)). The method of Lohmar et al. (1942) involving oxidation of a uronic acid to a dicarboxylic acid, with subsequent identification of the latter as the dibenziminazole derivative is open to the criticism that it will not distinguish between D-glucuronic and L-guluronic acids as both of these acids give D-glucosaccharic acid on oxidation. The method has however been employed by Levvy (1948) working with small quantities of menthyl glucuronide, and although Bernhauer and Irrgang (1935) believe that they have demonstrated the production of L-guluronic acid by the action of certain bacteria on glucose, this acid has not so far been found to be produced by animal organisms. It therefore appeared that this method would provide

a means of identifying the uronic acid moiety of oestriol "monoglucuronidic" acid with reasonable certainty.

The hydrolysis necessary for the liberation of the uronic acid from combination with oestriol presented certain difficulties. The work of Stephenson and Marrian (1947) on the acid hydrolysis of conjugated oestrogens in human pregnancy urine suggested that the complete hydrolysis of oestriol "monoglucuronide" in urine may require as long as 30-40 minutes boiling with 15% (v/v) of concentrated hydrochloric acid. It was felt that such harsh treatment might result in extensive destruction of the uronic acid liberated. Accordingly milder means of hydrolysis were sought.

Odell, Skill and Marrian (1937) and Fishman (1939) showed that preparations of β-glucuronidase obtained from animal tissues will liberate oestriol form sodium oestriol 'monoglucuronidate', and Levvy (1948) has shown that this enzyme will liberate glucuronic acid from menthyl glucuronide. These facts suggested that hydrolysis by the action of glucuronidase might be suitable for the present purpose.

(b) Preparation of β-glucuronidase.

The enzyme was prepared from ox spleen, and its activity determined by the procedures described by Graham (1946). On account of great losses of activity in the final stages, preparations were not purified beyond Graham's stage E.

(c) The hydrolysis of sodium oestriol 'monoglucuronidate' and isolation of the uronic acid as the dibenziminazole derivative of D-glucosaccharic acid.

350 mg. sodium oestriol 'monoglucuronidate' were incubated at 37° in 250 ml. 0.25M acetate buffer, pH 4.6, containing 1000 Graham units of β-glucuronidase. 1 ml. toluene was added to inhibit growth of bacteria. A further 500 units of enzyme in 45 ml. solution were added after 24 hours. At the end of 3 days protein was precipitated with 4 volumes of dry acetone, and filtered off. The filtrate was evaporated to a volume of 100 ml. It was then acidified with sulphuric acid and extracted three times with 30 ml. ether to remove oestriol. From its reducing power the ether extracted aqueous phase appeared to contain 87 mg. uronic acid, corresponding to 62% hydrolysis of the oestriol

'glucuronidate'. This uronic acid fraction was oxidised with bromine and the dibenziminazole derivative of the product was prepared by treatment with o-phenylenediamine in a yield of 47 mg. by the methods described by Lohmar et al. (1942) as modified by Levvy (1948).

The hydrolysis was repeated with a further 340 mg. sodium oestriol glucuronidate. In this case 79% hydrolysis was indicated and 55 mg. dibenziminazole derivative finally obtained. This was combined with the product of the first experiment before purification.

The dibenziminazole isolated had m.p. 242-243° (decomp.). The mixed m.p. with an authentic sample of dibenziminazole derivative of D-glucosaccharic acid (m.p. 241-243°, decomp.) was 242-243° (decomp.). The dipicrate and dihydrochloride were prepared as described by Levvy (1948). The dipicrate melted with decomposition at 210° after change of form at 142°; an authentic sample melted at 211° (decomp.), after change of form at 142°, and showed no depression in m.p. after mixture with the material isolated. The dihydrochloride melted at 265-266° and had $[\alpha]_D^{18} = +49.8° \pm 0.6°$ in water (c, 2.057). On admixture with an authentic sample (m.p.266-267°; $[\alpha]_D^{18} = +49.3° \pm 0.6°$ in water (c, 2.042)) the melting point was unchanged. Levvy (1948) found

$[\alpha]_D^{18}$ = + 52.3° in water (c, 2.024) with his preparation of the dihydrochloride, which he showed to be the tetrahydrate. In order to avoid difficulty with varying degrees of hydration in this work, both dihydrochloride isolated and authentic dihydrochloride were recrystallized from water, and dried over $CaCl_2$, under identical conditions.

(d) Conclusions.

The uronic acid fraction obtained from the products of enzyme hydrolysis of NaOG was oxidised and treated with o-phenylenediamine to give a product which was satisfactorily identified as the dibenzimin-azole of D-glucosaccharic acid. It is clear therefore that the uronic acid obtained by the enzymic hydrolysis was either D-glucuronic acid or L-guluronic acid. In view of the improbability that the latter is produced in animal organisms, it seems reasonable to assume that the uronic acid is indeed D-glucuronic acid. No evidence has been obtained regarding the ring structure or anomeric form of the uronic acid. Glucuronic acid is at present known to occur naturally only as the β-form.

8. Preliminary Investigation of the "Oestriol" Isolated from the Products of Enzymic Hydrolysis of NaOG.

One of the objects of this research was to investigate the purity of NaOG prepared and purified as thoroughly as existing methods permit. For this purpose the oestriol liberated from conjugation with glucuronic acid by the enzyme hydrolysis described in the previous section was isolated for further study.

(a) Isolation of Oestriol.

The combined ether extracts of the acidified hydrolysis products (Section 7(c) page 82) were washed four times with 25ml. volumes of 5% (w/v) $NaHCO_3$, three times with 25 ml. volumes of water, and evaporated to dryness. The nearly white solid residue weighed 289 mg. Treatment with charcoal in a minimum of hot methanol removed the slight colour from the solution. Evaporation of the methanol left a white solid. This will subsequently be known as the "isolated oestriol".

(b) Standard Oestriol for comparison with the isolated oestriol.

The "standard oestriol" provided by Professor G.F. Marrian was prepared by saponification

of recrystallized oestriol acetate. The material thus obtained was recrystallized twice from anhydrous methanol to give glass clear needles.

After drying for 6 hours over P_2O_5 in vacuo at 80° the m.p. in an evacuated capillary was 283-284°.

In this work it was often found advantageous to seal samples in capillaries evacuated to about 5 x 10^{-3} mm. Hg before determining the m.p. Oestriol for example melts in an open capillary with some decomposition yielding an amber coloured liquid, but melting takes place in an evacuated capillary at 1-2° higher temperatures to give an almost colourless liquid.

C and H analyses of the standard oestriol gave

C, 74.96, 75.01; H, 8.33, 8.23%.

Calc. for oestriol $C_{18}H_{24}O_3$

C, 74.96; H, 8.39%.

Specific rotation determined in a micro tube.

$$\left[\alpha\right]_D^{16.5^\circ} = + 51.0^\circ \pm 1.44 \qquad c = 0.7602 \text{ in ethanol.}$$

(c) Examination of Isolated Oestriol.

After drying in vacuo over $CaCl_2$ for several days at room temperature the isolated oestriol melted in an evacuated capillary at 283-285°.

C and H analyses gave C, 72.07, 71.86; H, 8.32, 8.37. Calc. for oestriol $C_{18}H_{24}O_3$ C, 74.96; H, 8.39.

Specific Rotations

$$\left[\alpha\right]_D^{19^\circ} = + 54.53 \pm 1.14^\circ. \quad c = 0.8435 \text{ in ethanol.}$$

The Oestriol content was determined by the quantitative Kober test employing the technique of Stephenson and Marrian (1947). No absorption was observed after fading the pink solutions obtained for 1.5 hours at 100°. The photo electric absorptiometer readings obtained before fading were converted to weights of oestriol by reference to a calibration curve prepared with the standard oestriol. Employing 20, 30 and 40 µg quantities of the isolated oestriol, oestriol contents were found to be

81.2, 87.8 and 83.7%.

A David Test (David 1934) was performed on the isolated oestriol and on the standard oestriol, employing approximately 20, 40 and 400 µg. of material.

The reagent was prepared by dissolving a minute crystal of A.R. ferric ammonium sulphate in 10 ml. conc. A.R. H_2SO_4. 1 ml. of this reagent was added to the dry residue of oestriol in a test tube. The tube was heated in a boiling water bath for 10 minutes, cooled in ice and water, diluted with 3 ml. water and reheated for 3 minutes to develop the colour.

The solution appeared blue by transmitted light, and showed a greenish fluorescence by reflected light. The intensity of the colour varied with the amount of oestriol used. Viewed with the unaided eye there was no obvious difference in shade of colour produced by the isolated oestriol and the standard.

Conclusion.

Although the m.p. of the isolated oestriol appears to be satisfactory, the results of analyses for C and H and oestriol content show that the substance is far from pure.

9. Investigation of the Impurities in Isolated Oestriol.

(a) Adsorption of Water of Crystallization by Oestriol.

It was not possible to investigate the water content of the original sample of isolated oestriol since most of the sample had already been subjected to chromatography and other experiments. A 10 mg. sample of standard oestriol was therefore dissolved in methanol containing 10% (v/v) water, evaporated to dryness in vacuo, and dried under conditions identical to those applied to the isolated oestriol. In this way the standard oestriol would have the same opportunity of picking up water as the isolated material.

Analyses gave	C, 74.46; H, 8.37.
Found for the standard oestriol before treatment with wet methanol.	C, 74.99; H, 8.28.
Found for isolated oestriol.	C, 71.97; H, 8.35.
Calc. for oestriol $C_{18}H_{24}O_3$.	C, 74.96; H, 8.39.

Conclusion.

It is unlikely that adsorbed water will account for the analysis results obtained with the isolated oestriol.

(b) Attempted Purification of Isolated Oestriol by Chromatography.

Peter Spence Alumina Type H was washed with dil. HNO_3 then with water until the pH of the suspension was 5 (glass electrode), and activated after drying by heating for 4 hours in vacuo at 230°. The product had an activity of II (Brockmann and Schodder (1941)). 7.5 g. of this alumina were packed wet with acetone in a tube of 16 mm. diameter fitted with a Pyrex Sintered Glass Disc (Porosity 1) and glass tap.

The acetone used was purified by refluxing with alkaline permanganate followed by drying with anhydrous potassium carbonate and subsequent distillation

over an efficient fractionating column.

Methanol was purified by refluxing with NaOH and drying with quick lime.

Pyridine was dried with fused barium oxide.

A solution of 256 mg. of isolated oestriol in 100 ml. acetone was poured onto the column of alumina, and the chromatogram was developed with acetone, methanol and methanol containing 5% (v/v) pyridine.

The fractions obtained are described below.

Chromatography of Isolated Oestriol.

Solvent - Acetone.

Vol. of Fraction. (ml.)	Wt. of Fraction. (mg.)	Remarks.
1. 100.	5.	Oily.
2. 50.	70.	White crystalline solid.
3. 50.	57.	
4. 50.	37.	
5. 50.	21.	
6. 50.	6.	
7. 50.	8.	
8. 50.	6.	
9. 50.	6.	
10. 50.	3.	
11. 50.	2.	
	221 mg.	

Chromatography of Isolated Oestriol (contd.)

Solvent - Acetone.

Vol. of Fraction. (ml.)	Wt. of Fraction. (mg.)	Remarks.
	221	
12. 50.	3.	
13. 50.	2.	
14. 50.	3.	Slightly gummy.
15. 50.	0.	
16. 50.	0.	
	229 mg.	

Solvent - Methanol.

1. 50.	8.	
2. 50.	3.	Gum which could
3. 50.	3.	be leached away
4. 50.	0.	leaving traces of
5. 50.	0.	solid which would
	14 mg.	not burn.

Solvent - Methanol/Pyridine.

1. 50.	4.	Long needles which disappeared(? deliquesced) on removing flask from pump after sucking off solvent. The same result was obtained on passing methanol/pyridine through a "blank" column of alumina.
2. 50.	5.	
	9 mg.	

After "drying" under conditions identical with those applied to the original isolated oestriol, the crystalline material eluted by acetone melted at 279-280° (evac. capillary).

Analyses gave C, 74.47, 74.84; H, 8.52, 8.39%
Calc. for oestriol $C_{18}H_{24}O_3$ C, 74.96; H, 8.39%.

Specific Rotation on dried sample.

$[\alpha]_D^{19} = + 52.45 \pm 3.84°$ (c = 0.8387 in ethanol).

Oestriol content on dried sample.

The oestriol content determined by the quantitative Kober method already described and using quantities of about 20, 30 and 40 μg. was found to be 97.5%, 102.1% and 100.8%. Mean 100.2%.

The gum eluted by methanol (approx. 12 mg.) resisted attempts at recrystallization.

The properties of standard oestriol were unaltered by identical chromatographic treatment. 35 mg. oestriol were used, and the same weight was eluted with acetone. No gum was eluted by methanol.

The properties of the different materials before and after chromatography are collected in Table 3 .

Conclusion.

It appears that impurities present in oestriol isolated from the products of enzymic

TABLE 3.

Oestriol Isolated from Products of Enzymic Hydrolysis of NaOG Compared with Standard Oestriol.

Material.	m.p.	Analyses. % C	% H	Specific Rotation in ethanol.	Oestriol Content. %
Isolated Oestriol.					
before chromatography.	283-285°.	71.97	8.35	α_D^{19} + 54.53 ± 1.14° c = 0.8435.	81.2, 87.0, 83.7.
after chromatography.	279-280°.	74.66	8.46	α_D^{19} + 52.45 ± 3.84° c = 0.8387.	97.5, 102.1, 100.8.
Standard Oestriol.					
before chromatography.	283-284°.	74.97	8.28	$\alpha_D^{16.5}$ + 51.0 ± 1.44° c = 0.7602.	
after chromatography.	283-284°.	75.0	8.30	α_D^{20} + 52.7 ± 1.05° c = 0.9100.	
Calc. for oestriol $C_{18}H_{24}O_3$.		74.96	8.39		

hydrolysis of "pure" NaOG may be removed by chromatography. A product giving C and H analyses results in fairly good agreement with oestriol ($C_{18}H_{24}O_3$), and containing very nearly 100% oestriol as determined by the Kober Reaction may be obtained by eluting the chromatogram with acetone.

(c) Isolation of a second batch of oestriol from products of enzymic hydrolysis for further study.

As most of the first preparation of "isolated oestriol" had been subjected to chromatography, a second preparation was made by hydrolysing 353 mg. NaOG with β-glucuronidase as already described. This yielded 110 mg. "isolated oestriol". Dried to constant weight over P_2O_5 at 80° in vacuo the m.p. was 280-281° (evac. capillary), found C, 72.69, 72.35; H, 8.24, 8.06%. Calc. for oestriol C, 74.96; H, 8.39%.

This material is obviously similar to the first preparation.

(d) Attempted Purification by solvent partitioning.

91.5 mg. isolated oestriol dissolved in 50 ml. N/10-NaOH was extracted 4 x 25 ml. ether. The ether was washed 4 x 10 ml. water and evaporated leaving 2 mg. white solid. The aqueous phase was

acidified to pH 1 and extracted with 4 x 50 ml. ether. The ether was washed with 10 ml. 5% (w/v) $NaHCO_3$ and 4 x 10 ml. water and evaporated to give72.5 mg. white solid. After drying to constant weight over P_2O_5 at 80° in vacuo this melted at 273-275°, found C, 73.78; H, 8.33, calc. for oestriol $C_{18}H_{24}O_3$ C, 74.96, H, 8.39%. There was no residue after combustion.

Conclusion.

The isolated oestriol is not purified by the solvent partitioning procedure employed.

(e) Attempted Purification of Isolated Oestriol by High Vacuum Sublimation.

A sample of the isolated oestriol was sublimed at 190-200° and 5 x 10^{-5} mm. Hg. The white crystalline sublimate melted at 278-281° with slight decomposition on a Kofler Type micro m.p. apparatus. Analyses of the sublimate without further drying gave C, 73.69; H, 8.23%. Calc. for oestriol $C_{18}H_{24}O_3$ C, 74.96; H, 8.39%.

Conclusion.

High vacuum sublimation of the isolated oestriol does not effect appreciable purification as judged by C and H analyses results.

(f) Isolated oestriol from products of acid hydrolysis of NaOG.

In order to investigate the possibility that the impurity in isolated oestriol is an artifact of enzymic hydrolysis, 50 mg. of the same batch of NaOG were subjected to acid hydrolysis. Possible Oxidative destruction of hydrolysis products was minimized by using freshly prepared constant boiling A.R. HCl and conducting the hydrolysis under a stream of nitrogen. The NaOG was dissolved in 72 ml. water, brought to boiling, 28 ml. constant boiling HCl added and boiling continued for 10 minutes. The cooled mixture was extracted with 6 x 25 ml. peroxide free ether. The ether was washed 2 x 15 ml. 5% (w/v) $NaHCO_3$ and 4 x 15 ml. water, and evaporated to give 21 mg. white solid (71% hydrolysis). After drying to constant weight as usual the m.p. was 283-284° (evac. capillary) and found C, 73.79; H, 8.63%. Calc. for oestriol $C_{18}H_{24}O_3$ C, 74.96; H, 8.39%.

Sublimation of a sample at 200° and 5 x 10^{-5} mm. Hg gave a product melting at 282-285° (evac. capillary), found C, 72.4; H, 8.11%.

Conclusion.

As in the case of enzymic hydrolysis, acid hydrolysis of "pure" NaOG followed by ether extraction

has produced oestriol with a fairly satisfactory m.p. but giving unsatisfactory C and H analyses figures.

(g) Extraction of Hydrolysis Products with Ethyl Acetate.

As ether may be an objectionable solvent for the extraction of oestrogens from the product of hydrolysis, the acid hydrolysis of NaOG was repeated with 46 mg. of the same batch of material. The hydrolysis mixture was adjusted to pH 7 and extracted with 50 ml. and four 25 ml. volumes of pure dry ethyl acetate. The acetate extract was washed once with 15 ml. 5% $NaHCO_3$ and three times with 15 ml. water and evaporated under reduced pressure. The residue weighed 22.4 mg. (82% hydrolysis). After drying to constant weight as usual, the m.p. was 283-285°, found C, 70.11; H, 8.79; calc. for oestriol C, 74.96; H, 8.39%.

Conclusion.

The material obtained by ethyl acetate extraction of the products of acid hydrolysis of "pure" NaOG does not appear to be purer than that obtained by ether extraction.

(h) Attempted Purification of Isolated Oestriol from Acid Hydrolysis by extraction with Hot Water.

The isolated oestriol extracted by ether from the products of acid hydrolysis of NaOG was leached with 2 ml. water at 100° for 5 minutes, and soluble material filtered off. The leaching was repeated two times exposing a fresh surface of the "oestriol" on each occasion by dissolving in ethanol and evaporating the solvent. In this way 13.5 mg. "oestriol" gave 5 mg. water soluble material.

The water insoluble fraction after drying to constant weight as usual melted at 283-284° (evac. capillary) and gave C, 74.11; H, 8.39%. Calc. for oestriol C, 74.96; H, 8.39%.

The water soluble fraction melted at 270-272° (evac. capillary) with decomposition (sudden frothing of the darkened material).

9 mg. of the oestriol standard treated with hot water in an identical manner gave about 0.7 mg. water soluble material which proved to be pure oestriol which had dissolved in the hot water.

The behaviour of the water soluble material from "isolated oestriol" suggested the presence of unchanged HOG, or possibly a decarboxylation product of HOG formed by the heating with HCl.

In order to investigate this possibility, the acid hydrolysis was repeated with 50 mg. of the same batch of NaOG, boiling for 90 minutes. Ether extraction gave 22 mg. white solid (74% hydrolysis). After drying in the usual manner the m.p. was 283-285°, found C, 74.08; H, 8.58%. Calc. for oestriol C, 74.96; H, 8.39%.

Leaching of 15 mg. with 3 x 2 ml. hot water as before gave 12.5 mg. water insoluble material which melted sharply at 285-286° and 2.3 mg. water soluble material which melted at 267-268° with decomposition (sudden frothing of the darkened material).

10. General Conclusions Regarding the Purity of NaOG.

NaOG prepared by the method of Cohen, Marrian and Odell (1936) slightly modified, and purified as thoroughly as existing methods and conditions permit, gives on enzymic or acid hydrolysis an oestriol fraction which is not pure oestriol. This "isolated oestriol" may be purified by chromatography but not by high vacuum sublimation nor by partitioning between ether and aqueous alkali.

There is at present no definite proof that the impurities in the products from enzymic and acid

hydrolyses are the same.

It appears that there is a water soluble impurity in the product from acid hydrolysis which is unlikely to be unchanged HOG but behaves similarly in the m.p. capillary. The product from enzymic hydrolysis has not been treated with hot water.

The low percentages of carbon found in the analyses of the isolated oestriol suggest the presence of some more highly oxygenated material, possibly a tetrahydroxy compound or a decarboxylated form of HOG.

The investigation has been hindered by lack of material. It is obviously desirable to repeat part of this work on a larger scale, particularly to attempt to isolate the impurity, and to investigate the purity of NaOG after repeated recrystallization.

11. Methylation of HOG.

Attempts to prepare a fully methylated HOG were undertaken as a preliminary to an attack on the problem of the position of attachment of the glucuronic acid to the oestriol. The intention was to prepare and hydrolyse a fully methylated HOG, and to remove the steroid hydroxyl group thus exposed, leaving an oestradiol dimethyl ether which could be identified.

(a) Preparation of HOG for Methylation.

Approximately 1.5 g. NaOG from various batches were dissolved in 800 ml. moist butanol and adjusted to pH 1 with HCl. The butanol solution was washed repeatedly with 50 ml. volumes of water until washings were free from chloride. The butanol was then evaporated under reduced pressure at 30° to give 1.02 g. nearly white solid. This was warmed with 10 ml. water and dried over $CaCl_2$ and subsequently over P_2O_5. A sample dried over P_2O_5 at 80° in vacuo for 15 hours gave C, 62.01; H, 7.02%. Calc. for HOG $C_{24}H_{32}O_9$ C, 62.03; H, 6.98%.

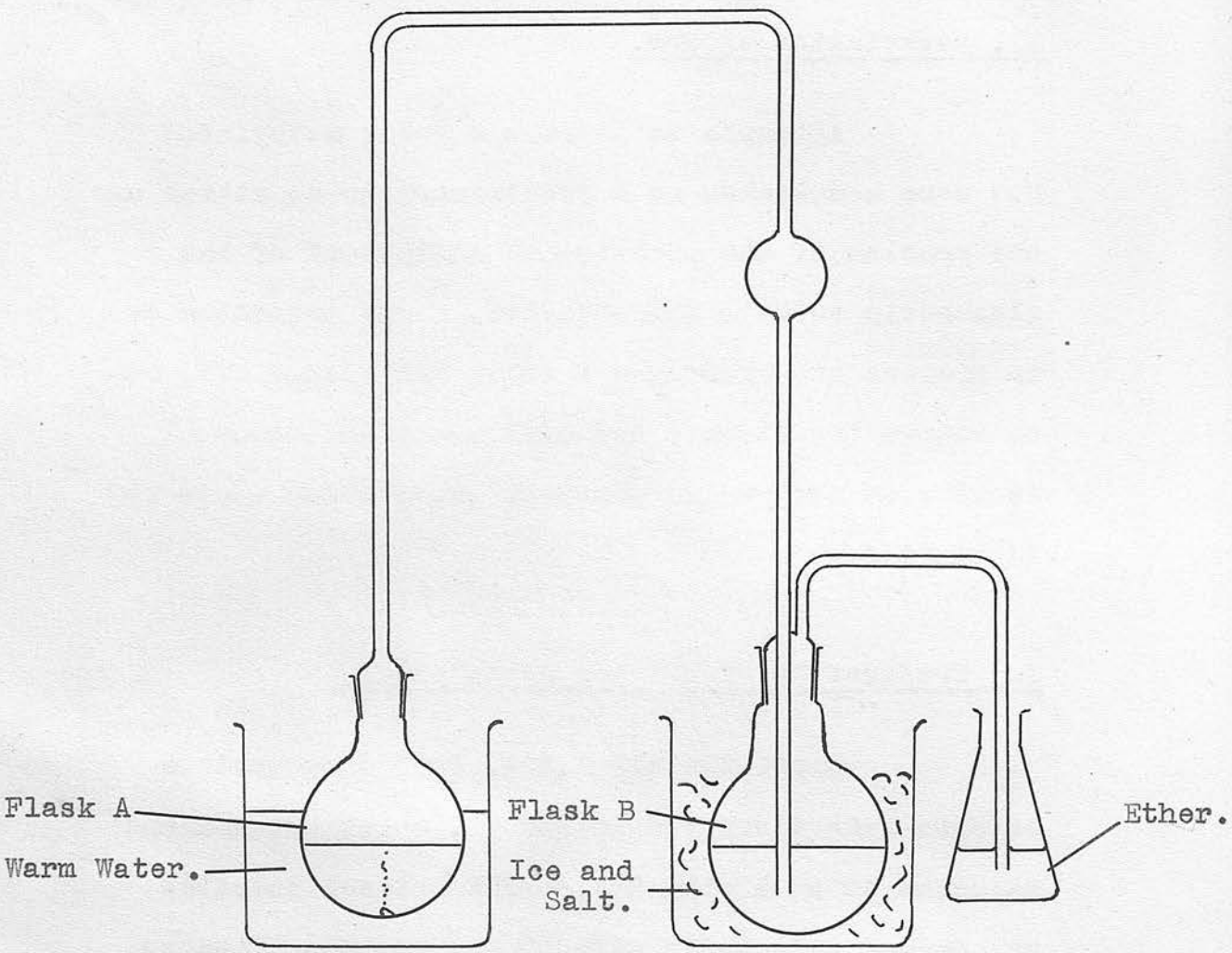


Fig. 10. Apparatus for methylation with diazomethane.

(b) Attempted Methylation with Diazomethane.

The HOG (0.9 g.) prepared as described above was dissolved in 70 ml. aldehyde free dry methanol in flask A (Fig.10). 5 ml. nitroso N methyl urethane, 50 ml. dry ether and 5.3 ml. 25% (w/v) KOH in methanol were placed in flask B. With flask A in ice and salt, the contents of B were heated gently in order to distill ether and diazomethane slowly into A. When about 2/3 of the ether had distilled a further 20 ml. were added and the distillation continued until the volume had once again been reduced to 1/3. The whole process occupied 25 minutes. Solvents were evaporated off the methylated HOG under reduced pressure, and the procedure was repeated a second time. 0.959 g. of light brown frothy solid remained. This was more soluble in methanol and much less soluble in water. After drying at room temp. in vacuo over P_2O_5 a sample melted in an evacuated capillery as follows - softening commenced at 100°, bubbles of gas appeared in the golden yellow mass at 162° followed by rapid darkening and decomposition at 215°. Found C, 62.69; H, 7.30; OCH_3 4.77. $C_{24}H_{31}O_8(OCH_3)$ requires requires C, 62.70; H, 7.16; OCH_3 6.49%.

A sample of the partly methylated material dissolved slowly (in a few hours) in 9% (w/v) Na_2CO_3

and gave a strongly positive Millon's Test in the cold.

About 0.5 g., dissolved in ethanol, coupled with diazotized sulphanilic acid to give a red colour. (Schmulovitz and Wylie (1936)).

These results indicate the presence of a free phenolic hydroxyl and the probable presence of a methyl ester. Failure to methylate the phenolic hydroxyl could not be explained at the time and the bulk of the partly methylated material was acetylated as described later.

Several months later when a fresh supply of nitroso N methyl urethane became available 200 mg. of HOG was subjected to the same methylation procedures and gave 212 mg. of frothy gum. This was very soluble in acetone and in benzene. The benzene solubility is remarkable in view of the insolubility of HOG in benzene. The material was almost insoluble in ether, sparingly soluble in cold, but more soluble in hot ethyl acetate.

Chilling a hot saturated solution in ethyl acetate gave a semi-crystalline gel. The mother liquors were poured off the gel and the "crystallization" was repeated. In this way 62 mg. of material were obtained. This melted at 135-144° (evac. capil.). The m.p. was unaffected after drying 18 hours at 80°

in vacuo over P_2O_5. Analyses of the dried material for methoxyl content gave 12.2 and 12.6% (mean 12.4%); oestriol glucuronidic acid monomethyl ether methyl ester, $C_{23}H_{30}O_5(OCH_3)COOCH_3$ requires 12.6% methoxyl. No reaction was obtained with Millon's Reagent and no pink colour appeared on attempting to couple the substance with diazotized sulphanilic acid. The substance was insoluble in Na_2CO_3 solution. These observations support the view that the substance is the 3 methyl ether methyl ester of HOG.

(c) Attempted Methylation using Methyl Iodide and Silver Oxide.

Preliminary experiments were carried out with oestriol

Preparation of Oestriol 3 methyl ether.

110 mg. oestriol was methylated once with diazomethane by the procedures already described to give 119 mg. of faintly blue crystalline solid. Observed on a Kofler Type micro m.p. apparatus the crystals started to soften at 150° and melted to a mobile liquid at 175°. In an open capillary softening commenced at 152° and the whole mass was liquid at 182°. Two old samples of oestriol monomethyl ether provided by Professor Marrian behaved in the same way softening at about 150° and clearing at 160°. No sharp m.p. was obtained. There was no improve-

ment of the freshly methylated oestriol after recrystallization from aqueous ethanol. Methoxyl content was not determined at this stage. The product was probably oestriol 3 methyl ether.

Further Methylation with MeI and Ag_2O.

119 mg. of the material methylated with diazomethane were dissolved in 5 ml. anhydrous methanol. 10 g. redistilled MeI and 1 g. dry Ag_2O were added and the mixture was heated under a reflux condenser for 8 hours and stood overnight in the laboratory. Insoluble material was filtered off and washed several times with boiling methanol. The filtrate and washings were evaporated under reduced pressure to give 118 mg. nearly white solid. The methylation was repeated with 18 hours boiling as before but using only 0.5 g. Ag_2O. The product was a golden yellow gum weighing 125 mg. After a third methylation the weight of the gum had not changed. The gum was dissolved in 5 ml. ethanol, boiled with charcoal and filtered. The residue after removing the ethanol was distilled at 195° and 4×10^{-4} mm. Hg to give an oily distillate. This set to a hard partly crystalline mass. It was recrystallized by dissolving in 2 ml. boiling chloroform and adding

hexane carefully until the solution became cloudy, followed by boiling to clear the solution and refrigeration. In this way 48 mg. pale grey-green crystals were obtained. The recrystallization was repeated using acetone and hexane. 7 mg. almost white crystals were obtained. These melted at 128-129° on the hot stage micro melting point apparatus. Carbon, hydrogen and methoxyl analyses gave

C, 75.84; H,8.73; $-OCH_3$ 19.2%.

oestriol trimethyl ether $C_{18}H_{21}(OCH_3)_3$ requires — C, 76.50; H, 9.22; $-OCH_3$ 28.2%.

oestriol dimethyl ether $C_{18}H_{21}(OCH_3)_2OH$ requires — C, 76.00; H, 8.91; $-OCH_3$ 19.6%.

The substance was probably oestriol dimethyl ether.

(d) Attempted Methylations using Thallium Ethoxide.

Since it appeared that Methyl Iodide and Silver Oxide would not readily give an oestriol trimethyl ether, and might therefore fail to give a completely methylated HOG, experiments were performed using thallium ethoxide kindly supplied by Dr. R.C. Menzies of the Chemistry Department, Edinburgh University.

In order to investigate the possibility that the steroid A ring might be oxidized when using

this reagent, attempts were first made to methylate oestrone.

Methylation of Oestrone with Thallium Reagent.

100 mg. oestrone, m.p. 266-267°, were dissolved in 10 ml. ethanol-benzene 50% (v/v) and the solution was taken to dryness under reduced pressure twice with 0.1 ml. thallium ethoxide. The residue was refluxed for 96 hours in the dark with 12 ml. MeI and 1 ml. methanol, with precautions to exclude moisture and CO_2. After filtering off the thallous iodide and evaporating the filtrate, the residue was distilled at 155° and 1 x 10^{-4} mm. Hg. The distillate, 93.7 mg. white crystalline solid, melted at 168-171.5°. Butenandt (1932) reported 168.5-169° for the m.p. of oestrone methyl ether. One recrystallization from ethanol gave 75 mg. white crystals m.p. 169-171°, containing 10.80% methoxyl. Oestrone monomethyl ether $C_{18}H_{21}O(OCH_3)$ requires 10.90%.

Obviously there is no extensive oxidation of the steroid A ring using this thallium reagent.

Methylation of Cholesterol with Thallium Reagent.

In order to investigate the ability of this reagent to methylate secondary alcoholic hydroxyls as found in the steroid molecule, an attempt was made to prepare cholesterol 3 methyl ether.

100 mg. cholesterol were dissolved in 5 ml. ethanol-benzene (50% v/v). The solution was evaporated to dryness under reduced pressure with 0.1 ml. thallium ethoxide. The residue was refluxed for 96 hours in the dark with 10 ml. MeI taking precautions to exclude moisture and CO_2. Evaporation of the filtrate from the thallous iodide gave a nearly white solid. Recrystallization of this from methanol gave 81 mg. white crystalline solid which melted at 82.5-83° and contained 7.3% methoxyl. Calc. for cholesterol monomethyl ether, $C_{27}H_{45}OCH_3$, 7.73% methoxyl.

Secondary alcoholic hydroxyl groups appear to be readily methylated.

Attempted Methylation of Oestriol using Thallium Reagent.

97 mg. oestriol were dissolved in 5 ml. ethanol-benzene (50% v/v) and the solution was twice evaporated to dryness under reduced pressure with 0.1 ml. thallium ethoxide. The residue was a slightly gummy, almost white, frothy solid. This was refluxed in the dark with 10 ml. redistilled MeI and 1 ml. anhydrous MeOH. The contents of the flask were protected from moisture and CO_2. After 90 hours the amber coloured solution was filtered from thallous iodide. The filtrate was boiled with a small amount of charcoal, filtered, and evaporated under reduced

pressure to give a yellow gummy solid. This distilled at 150° and 4 x 10^{-5} mm. Hg to give a greenish yellow oil which solidified on cooling. The distillate was crystallized twice from ethanol containing 10% (v/v) hexane to give 18 mg. of faintly greenish blue crystals. These melted at 148-152° after preliminary softening at 140°. On admixture with authentic oestriol 3 monomethyl ether (m.p. 150-155°) the m.p. was 148-153°. After drying at 80° in vacuo over P_2O_5 for 2 hours the methoxyl content was found to be 10.8%. Calc. for oestriol monomethyl ether $C_{18}H_{21}(OH)_2OCH_3$ - 12.9%.

Methylation of oestriol making use of thallium ethoxide thus appears to give a product which is probably a monomethyl ether only.

(e) Acetylation Experiments.

In view of the failure to obtain a fully methylated oestriol derivative by the silver oxide and thallium ethoxide methods, and the fact that Cohen, Marrian and Odell (1936) obtained oestriol 3 monomethyl ether alone from the products of hydrolysis of NaOG methylated with methyl sulphate, it appeared likely that a fully methylated NaOG would not be readily obtained by direct methylation procedures. Attention

was therefore turned to the possibility of acetylating the material first and then treating the product with NaOH and methyl sulphate under conditions which would be expected to replace acetyl groups by methoxyl groups. This technique has been used in the case of certain polysaccharides which have proved difficult to methylate by other means. (personal communication from Professor E.L. Hirst).

Acetylation of Partly Methylated HOG.

950 mg. of the product described in section 11(b) (page 102) which appeared to be HOG methyl ester, were dissolved in 100 ml. acetic anhydride and 5 ml. pyridine. After heating the solution for 4 hours at 110° the acetic anhydride and pyridine were removed under reduced pressure, leaving a dark brown mass. After storage for several weeks in vacuo over $CaCl_2$ and KOH pellets this product weighed 1.35 g. It dissolved readily in 50 ml. hot methanol and formed white crystals on chilling the solution. The crystals were filtered off and washed with cold methanol. After a second crystallization from methanol 541 mg. crystals were obtained. These melted to a straw coloured liquid at 164-165°. Analyses gave C, 59.77; H, 6.20%. HOG penta acetate methyl ester

$C_{23}H_{26}O_7(CH_3CO)_5COOCH_3$ requires C, 61.02; H, 6.44%.

The material was readily soluble in cold acetone, chloroform, benzene and ether, sparingly soluble in ethanol and almost insoluble in hexane. On cooling a hot saturated solution in ethanol the whole solution set to a stiff gel. Six recrystallizations from methanol did not raise the m.p. The m.p. was also unchanged after sublimation of the crystals in high vacuum. Millon's reaction was negative and no colour was obtained on attempting to couple the material with diazotized sulphanilic acid. Analyses of the repeatedly recrystallized material gave mean values of C, 60.47; H, 6.76; $-OCH_3$, 4.33%. HOG pentaacetate methyl ester $C_{23}H_{26}O_7(CH_3CO)_5COOCH_3$ requires C, 61.02; H, 6.44; $-OCH_3$ 4.5%.

It appears likely that this material is the pentaacetate methyl ester of HOG containing smaller amounts of closely related substances not readily removed by recrystallization. It is possible that the partly methylated HOG used as the starting material may have been a mixture of the methyl ester with smaller amounts of the 3 methyl ether. The methyl ether methyl ester obtained on the second attempted methylation of HOG with diazomethane was not available for acetylation.

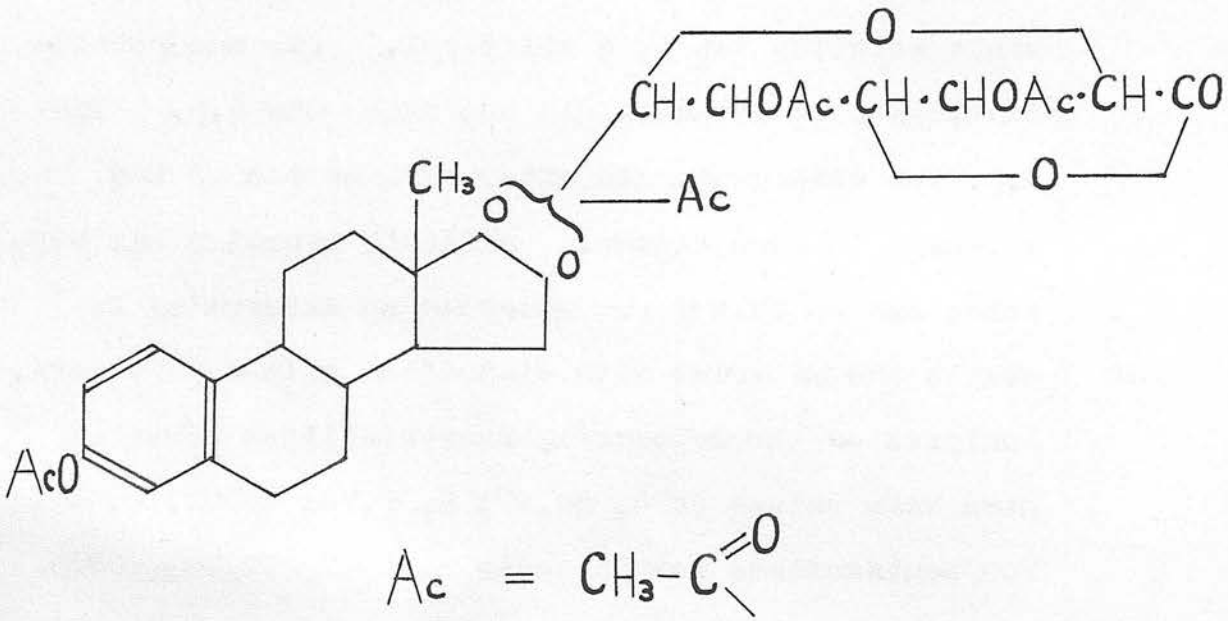


Fig. 11. Oestriol glucuronidic acid tetraacetate lactone.

Acetylation of NaOG.

83 mg. NaOG were warmed at 60° for 2 hours in 10 ml. acetic anhydride and 2 ml. pyridine. After standing at room temperature for a further 60 hours the solution was diluted with 100 ml. water and extracted 4 times with 25 ml. ether. The ether extract was washed successively with 3 x 10 ml. volumes 2N-HCl, 3 x 10 ml. volumes of N-NaOH and 5 x 10 ml. volumes of water, and evaporated under reduced pressure to give 96 mg. white solid. This was recrystallized three times by dissolving in a minimum of boiling methanol, adding water until the solution became cloudy, boiling to clear the solution and cooling slowly. Clusters of short, thick, glass clear needles were obtained. After drying to constant weight at 110° the m.p. in an open capillary was 204-205° without decomposition, found C, 62.38; H, 6.15%. Oestriol glucuronidic acid tetraacetate lactone requires C, 62.52; H, 6.23%. The probable structure of this substance is shown in Fig. 11.

12. General Conclusions Regarding Methylation Experiments.

The experiments reported in this section can only be regarded as a preliminary investigation of the possibility of obtaining a fully methylated HOG.

In view of the failure to obtain fully methylated oestriol by means of the methyl iodide silver oxide or thallium ethoxide methods and the fact that Cohen, Marrian and Odell (1936) obtained the 3 methyl ether alone from the hydrolysis products of NaOG treated with methyl sulphate it seems unlikely that a fully methylated derivative will be obtained by direct methods.

The failure to prepare oestriol trimethyl ether may be due to the spatial arrangement of the C_{16} and C_{17} hydroxyls and C_{13} angular methyl group in the oestriol molecule hindering the approach of the methylating reagent. This arrangement is however no barrier to the formation of oestriol triacetate (Thayer et al. (1931)) or oestriol glucuronidic acid tetraacetate lactone as described above.

No satisfactory explanation can be offered for the failure to obtain the methyl ether methyl ester of HOG in the first experiment with diazomethane. As further supplies of the nitroso N methyl urethane

reagent were not available at the time the attempts to acetylate the partly methylated HOG were started. At a later date, when the reagent required for the preparation of the diazomethane became available again, a second, smaller batch of HOG gave a satisfactory 3 methyl ether methyl ester derivative.

Further investigations were postponed until larger quantities of NaOG could be made available. When such quantities are available, if it is still found impossible to methylate the HOG by way of the fully acetylated derivative, it may be possible to obtain hydrolysis conditions which may permit the splitting of the linkage between the oestriol and glucuronidic acid in a fully acetylated HOG without removing acetyl groups. Such an approach would, in addition, require an investigation of the stability of oestriol acetates under the conditions required to remove the oestriol hydroxyl group involved in the link with the glucuronic acid. The preparation and properties of the three oestriol acetates have not been thoroughly investigated.

PART III.

A SEARCH FOR POSSIBLE METABOLIC PRODUCTS OF PROGESTERONE IN THE URINE OF GOATS RECEIVING PROGESTERONE.

1. Introduction.

The excretion in the urine of metabolic products of progesterone by the goat has not been investigated, and reports of a possible species specificity in the excretion of pregnanediol have appeared in the literature (see Section 2). In view of these facts it appeared to be of interest to examine urine from goats receiving large amounts of progesterone. An opportunity to examine such urine arose during lactation experiments with goats at the National Institute for Research in Dairying.

The results of this work are reported in this Part of the Thesis.

2. Isolation Studies in the investigation of the metabolism of progesterone.

Numerous investigators have speculated on the possible interconvertions of steroid hormones within the body. Experimental support for some of their views has come from the results of exhaustive isolation studies in which steroid excretion products have been obtained in crystalline form. Pregnancy urine from humans and other mammalian species has proved a particularly rich source of these steroids. In other cases products isolated from urine after the administration of pure steroid hormones have provided valuable information.

As pointed out in Part I of this thesis the metabolism of progesterone has been the subject of numerous excellent reviews. Only brief reference will therefore be made to earlier work in this introduction to a study of the urine of goats receiving large doses of progesterone.

Since the isolation of pregnane-3α:20α-diol from human pregnancy urine by Marrian in 1929 a number of closely related steroids, mostly reduced forms of progesterone, have been obtained from urine. The more common of these are listed in Table 4. Pregnanediol alone has been isolated in considerable quantities after the administration of progesterone

TABLE 4

ISOLATION OF C_{21} STEROIDS RELATED TO PROGESTERONE FROM URINE.

Steroid.	Source.	Investigator.
Pregnanedione-3, 20.	Pregnant Mare Urine.	Marker et al. (1937).
Allopregnanedione-3,20.	Pregnant Mare Urine.	" "
Pregnane-3α-ol-20-one.	Pregnant Human Urine.	Marker & Kamm. (1937).
Allopregnane-3α-ol-20-one.	" "	Marker et al. (1937).
Allopregnane-3β-ol-20-one.	" "	Pearlman et al. (1942).
Δ^5pregnen-3β:20α-diol.	" "	Marker & Rohrmann. (1938).
Pregnane-3α:20α-diol.	" "	Marrian. (1929).(b)
Pregnane-3β:20α-diol ?	Human Urine. (adrenal tumour case).	Mason & Kepler. (1945).
Allopregnane-3α:20α-diol.	Pregnant Human Urine.	Hartmann & Locher. (1935).
Allopregnane-3β:20α-diol.	" "	Marker & Rohrmann. (1939).
Pregnane-3α-ol.	" "	Marker & Lawson. (1938).

(Heard (1941)). This may be ascribed to the low solubility of pregnanediol in organic solvents and to the fact that it is readily extracted as the glucuronide from human urines. The available evidence however favours the belief that this pregnanediol is the main metabolic end product of progesterone metabolism in man.

Apart from pregnane 3α:20α-diol and pregnan-3α-ol-20-one there is no clear cut evidence that the other steroids listed in Table 4 are derived directly from progesterone. It is of course possible that these steroids may be derived from substances other than progesterone. Thus Cuyler, Ashley and Hamblen (1940) succeeded in isolating pregnanediol glucuronide in good yield from the urine of a man receiving desoxycorticosterone acetate, and pregnanediol itself has been isolated in other cases. Pregnanediol has also been obtained by Pearlman and Pincus (1946) after administration of Δ^5 pregnen-3β-ol-20-one, although this substance might well be a precursor of progesterone.

A review of the results of isolation studies appears to indicate that a species difference exists in the excretion of pregnanediol and its isomers (Pincus and Pearlman (1945)). Pregnanediol glucuronide has been isolated only from human urines

and from the urine of rabbits receiving progesterone. The species difference may however be more apparent than real. Failures to detect pregnanediol in urine may have been due as much to the inadequacy of methods employed as to the low concentration or absence of this substance. (Verly, Sommerville and Marrian (1950)).

3. Search for Progesterone Metabolites in Goat Urine.

(a) Experimental Animals - Urine Collection.

The experimental animals were mature ovariectomised goats on a diet of hay and a mixture of bran, linseed cake, oats and beans. For the purpose of lactation studies directed by Dr. S.J. Folley at the National Institute for Research in Dairying, Reading, they received by injection 1 mg. hexoestrol and 40 mg. progesterone daily over a period of 3 months. The first urine collection commenced 6 weeks after the start of hormone treatment.

Urine was collected quantitatively under butanol. Daily collections were stored in the refrigerator, and pooled 7 day collections were sent

to Edinburgh by passenger train. The weather was very cold at the time (Winter 1946-47) and it was unlikely that the temperature of the urine ever rose much above 10°.

(b) Extraction of Urine.

22 litres of urine collected on 34 days during which the goat received a total of 34 mg. hexoestrol and 1.3 g. progesterone were extracted in batches of about 5 litres. The pH was adjusted to 3 with HCl and the urine was extracted with 4,1/4 volumes n-butanol which had previously been purified by refluxing with NaOH and distilling. The butanol extract was washed twice with 1/10 volumes water and evaporated under reduced pressure to a small volume. These concentrates were stored in the refrigerator until the 22 litres had been extracted. During storage in the cold a considerable amount of white solid separated. A small quantity was leached with cold ethanol to remove brown contaminating material and recrystallized three times from hot water. The white crystalline product melted at 187°. Hydrolysis and extraction with ether yielded a white solid which, after recrystallization from water,

melted at 120° and did not depress the m.p. of a specimen of pure benzoic acid. The white solid present in the urine extract was probably hippuric acid (m.p. 187°).

The concentrate from the butanol extract of the goat urine was dissolved in 3 litres of hot water, heated to boiling and 450 ml. conc. A.R. HCl added through the reflux condenser fitted to the flask. The boiling was continued for 30 mins. After cooling, the hydrolysed mixture was extracted with 1000 ml. and five 700 ml. volumes of peroxide free ether. The ether was extracted with four 700 ml. volumes 5% (w/v) $NaHCO_3$. This $NaHCO_3$ extract ("acid fraction") was discarded. The ether was then extracted with four 700 ml. volumes N-NaOH. The aqueous extract was adjusted to pH 3 with HCl and extracted with six 700 ml. volumes of peroxide free ether. The ether was washed three times with 400 ml. water and evaporated to give a dark brown viscous gum. ("phenolic fraction").

The NaOH extracted ether phase was washed twice with 500 ml. water and evaporated to give a viscous ruby red gum. ("neutral fraction").

(c) Examination of "Neutral Fraction".

Distillation of the "neutral fraction" for 4 hours at 100-110° and about 1 mm. Hg pressure gave about 4 g. colourless liquid which appeared to consist mainly of a mixture of esters. The undistilled residue weighed 7.6 g. This was heated under reflux with 10.5 g. Girard's Reagent T (Girard and Sandulesco (1936)) dissolved in 50 ml. ethanol and 3.8 ml. glacial acetic acid. After 2 hours the mixture was cooled to 0° and diluted with 500 ml. ice cold water containing 35.3 ml. 1.7N-NaOH. This solution was then extracted six times with 100 ml. volumes of ether to remove non ketonic substances. The ether extraxt was washed once with 100 ml. 5% (w/v) $NaHCO_3$, and three times with 100 ml. water and evaporated to give 6.9 g. dark red gum ("non ketonic neutral fraction").

In order to free ketonic substances from combination with the Girard Reagent, 60 ml. conc. HCl were added to the ether extracted aqueous phase. After standing for one hour the mixture was extracted six times with 100 ml. ether. The ether was washed once with 100 ml. 5% (w/v) $NaHCO_3$ and three times with 100 ml. water and evaporated to give 0.29 g. gum ("ketonic neutral fraction").

(d) Examination of "Ketonic Neutral Fraction".

A chromatogram column was prepared from 9 g. Merck Alumina (Brockmann Activity II)[x] packed wet with benzene. The final dimensions of the column were 55 x 15 mm. The 0.29 g. red brown gum-Ketonic fraction - was dissolved in 10 ml. benzene and poured onto the column of alumina. The chromatogram was developed as follows:

No. and Vol. of fractions. (ml.)	Solvents.	Wt. of material recovered. (mg.)	Remarks.
12 x 10	benzene.	53.	Yellow gum.
13 x 10	1:1 benzene :ether.	65.	Yellow gum.
7 x 10	ether.	1.	Yellow gum.
13 x 10	1:1 ether : :acetone.	13.	Red gum.
10 x 10	methanol.	56.	Red brown gum.

Total wt. recovered 188 mg.

It was not found possible to crystallize any of the fractions.

x Brockmann and Schodder (1941).

(e) Examination of "Non Ketonic Neutral Fraction".

Distillation of this fraction for 13 hours at 110 - 115° and 10^{-4} mm. Hg gave about 4 g. of a reddish viscous oil. The residue, weighing 2.1 g., was heated on a boiling water bath for 2 hours with 1 ml. dry pyridine and 0.5 g. succinic anhydride. After cooling, the mixture was taken up in 50 ml. ether and extracted twice with 25 ml. 5N-H_2SO_4 and four times with 25 ml. N-Na_2CO_3. The ether was finally washed twice with 25 ml. water and evaporated to dryness giving 1.2 g. - "non carbinol, non ketonic neutral fraction".

The Na_2CO_3 extract containing the carbinols was acidified with H_2SO_4 and extracted with 100 ml. and three times with 50 ml. ether. The ether was washed once with 50 ml. 5% (w/v) $NaHCO_3$ and twice with 25 ml. water and evaporated to dryness. The residue was heated under reflux on a boiling water bath with 2 g. NaOH in 5 ml. water and 15 ml. ethanol. After 30 mins. most of the ethanol was removed under reduced pressure, the solution was diluted with 50 ml. water and extracted four times with 50 ml. ether. The ether was washed twice with water and evaporated to give 0.55 g. "carbinol non ketonic neutral fraction".

A chromatogram column was prepared from 24 g. Merck Alumina (Brockmann Activity II) packed wet with

benzene. The final dimensions of the column were 100 x 20 mm. The 0.55 g. brown gummy "carbinol, non ketonic neutral fraction" was dissolved in 10 ml. benzene and poured onto the column. The chromatogram was developed as follows:

No. and Vol.(ml.) of fractions.	Solvents.	Wt. (mg.) of material recovered.
9 x 10	Benzene.	7.
9 x 10	1:1 Benzene:Ether.	12.
12 x 25	" "	19.
10 x 25	Ether.	6.
17 x 25	1:1 Ether:Acetone.	201.
19 x 25	Acetone.	184.
17 x 25	Methanol.	183.

Total Weight of Material Recovered 612 mg.

All fractions were in the form of gums and, with the exception of the fraction eluted by acetone, resisted all attempts to crystallize them. On cooling for several days at 0° the "acetone fraction" solidified to pale pink crystals. After treating with charcoal and recrystallizing from aqueous ethanol 110 mg. white crystals melting at 50-52° were obtained. These smelt strongly of cats. In the sodium fusion test for elements N, S and halogens were absent. Phosphorus was also absent. Analyses

after drying for 48 hours in vacuo over P_2O_5 gave C, 62.4; H, 10.1%. The material sublimed readily at 110° and 20 mm. Hg. The sublimate melted at 54-56° after preliminary softening at 51°.

Owing to lack of material further investigations of the nature of this substance could not be pursued.

(f) Examination of Urine from a Goat Receiving Progesterone by the Procedure of Sommerville, Gough and Marrian for the Determination of Pregnanediol.

9.15 l. urine were collected over a period of 5 days from a goat which was receiving 1 mg. hexoestrol and 40 mg. progesterone daily, by injection, the treatment having started several weeks before the urine collection commenced. A sample of 500 ml. was made neutral to litmus and subjected to the procedures of Sommerville et al (1948) for the determination of pregnanediol in urine. Dr. Sommerville, who carried out this part of the work, reported that he could detect no pregnanediol in the sample.

(g) Search for Conjugated Substances Similar to Pregnanediol Glucuronide in the Urine from a Goat Receiving Progesterone.

The bulk of the urine collection referred to in the previous paragraph (ca. 8.5 l.) was extracted with butanol as described in paragraph (b) page 120 . The butanol extract was washed three times with 1/10 volumes of 0.3N-NaOH and once with 1/10 volume of water, allowing the two phases to clear before separating on each occasion. The washed butanol was evaporated to dryness under reduced pressure. The light brown solid residue (3.74 g.) was dissolved in 20 ml. 50% (v/v) acetone:water with warming and filtered hot. 200 ml. cold, dry acetone were added. After thorough mixing and refrigerating overnight the solution became cloudy and deposited a small amount of white solid. This solid did not melt at temperatures up to 300° and would not burn when heated on a spatula. It was probably inorganic material.

The results of this experiment gave no indication of the presence of NaPG nor of pregnanediol like glucuronides.

4. Conclusions.

The investigation of urine from ovariectomised goats receiving daily injections of 1 mg. of hexoestrol and 40 mg. progesterone, by the usual chemical methods, has failed to produce any crystalline material likely to be of steroid nature. The carbinol non ketonic neutral fraction of the ether soluble hydrolysis products gave a white crystalline solid melting at 50-52°. The nature of this substance, which did not appear to contain elements other than C, H and O, was not fully investigated owing to lack of material.

The results of this work cannot be regarded as conclusive. Since the investigation was terminated early in 1947 considerable experience has been gained in the methods of working up urines in this type of experiment. Verly, Sommerville and Marrian (1950) have shown that 10 mins. boiling with 0.1 vol. of conc. HCl gives optimum hydrolysis of the conjugated pregnanediol in rabbit urine, as it does in human urine. Optimum hydrolysis conditions were not determined for the goat urine and it is possible that the 30 mins. boiling with acid to which it was subjected may have destroyed much of the pregnanediol present. Too much weight cannot be placed on the failure to detect pregnanediol by the procedures described by Sommerville

et al (1948), since it was not shown that the goat urine extract was free from substances which would interfere with the Astwood and Jones (1941) procedure involved.

Failure to isolate pregnanediol glucuronide in these experiments cannot be taken as evidence that this substance was not present. The isolation of this glucuronide is beset with considerable difficulty. Heard et al (1941) ascribed the failure of their early attempts to isolate pregnanediol glucuronide from the urine of rabbits to the presence of impurities. More recently in this laboratory Mr. I. Kyle (unpublished observations) has found that unsatisfactory gummy products are obtained on attempting to precipitate pregnanediol glucuronide from human pregnancy urine extracts with acetone, if the extracts have been stored for any length of time before working up.

The presence of considerable quantities of p-ethylphenyl sulphate (to be described later) in the extracts may have interfered with the isolation of conjugated products of progesterone metabolism.

A more thorough investigation of the metabolism of progesterone in the goat was not proceeded with at the time, on account of the fact that very promising results were being obtained with

human subjects[x] and it was considered advisable to reserve very limited supplies of pure pregnanediol glucuronide and other standard substances for this work.

x See Sommerville (1948).

PART IV.

p-ETHYLPHENYL SULPHURIC ACID

IN GOAT URINE.

(1)Metabolic Products Containing Sulphuric Acid.

It has been known for over 50 years that vertebrates excrete a variety of phenolic substances in the urine as esters of sulphuric acid. The classical researches of Baumann during the latter part of the 19th Century on the excretion of phenols were a development of his work on the sulphates in urine. Investigating the indigo forming substances in urine Baumann (1876 (a)) found that they could be decomposed with the formation of sulphuric acid. Later (Baumann 1876 (b)) he showed that indoxyl occurs in the urine as the sulphuric acid ester. A phenolic ester isolated from horse urine was shown to be phenylsulphuric acid (Baumann 1878-79).

In general the substances excreted in the urine in conjugation with sulphuric acid may arise from the products of bacterial action in the intestines, as in the case of the simple phenolic substances such as p-cresol, or they may be formed in response to the administration of substances which may or may not be regarded as physiological. In addition to these exogenous substances, there are others of more strictly endogenous origin, which have been found in urine in conjugation with sulphuric acid. These are known to include certain steroids and possibly adrenalin.

The isolation of oestrone sulphate from mare's urine (Schachter and Marrian 1938) was the first evidence that substances of strictly endogenous origin are excreted in conjugation with sulphuric acid.

The same substance has been obtained more recently from stallions' urine (Jensen et al (1945)). Marrian (1937) suggested that the oestrone in human urine might be found in conjugation with sulphuric acid. Butenandt and Hofstetter (1939) did not isolate oestrone sulphate from human pregnancy urine, but on the basis of comparisons of the chemical and physical properties of their urine fractions with synthetic oestrone sulphate they deduced that oestrone was excreted as the sulphate by humans. From the results of in vitro experiments Crepy (1946) concluded that conjugation took place between oestrone and a substance similar to sulphuric acid in the liver.

Using a sulphatase preparation which they consider to be free from glucuronidase or general phenol esterase and specific as a phenol sulphatase Cohen and Bates (1949) have hydrolysed the conjugated oestrogens in pregnant women's urine. They consider that from 5 to 89% of the oestriol fraction and from 8 to 100% of the oestrone and oestradiol

fraction are conjugated with sulphuric acid. With improved fractionation procedures and methods of determining the oestrogens, and using highly purified enzymes this approach should yield results of great interest regarding the relative quantities of conjugation of these phenolic steroids.

The sulphuric acid esters discussed so far have been those derived from phenolic substances. A second important group of sulphuric acid esters occurring in urine are those "alcoholic sulphates" formed by certain non phenolic steroids which may be regarded as metabolites of true endogenous origin. It is an instance of our lack of knowledge in this field that although androgen sulphates have been isolated from urine, there is no evidence that such substances are formed after the administration of androgens. It is probable that no attempt has been made to find such substances in the numerous experiments reported involving the administration of androgens to animals and humans.

Androsterone sulphate has been found in human urine by Venning et al (1942), and Munsen et al (1942) have isolated dehydroepiandrosterone sulphate from the same source. The following steroids have been isolated from pregnant mare's urine in conjugation with sulphuric acid.

Δ^{16}allopregnen-3β-ol-20one (Klyne et al (1948)), allopregnane-3β-ol 20 one (Paterson and Klyne (1948)) and possibly uranediol (Klyne (1948)).

Whereas much time has been devoted to the study of the metabolism and excretion of administered substances (See Williams 1947), as yet insufficient attention appears to have been paid to the isolation of conjugated substances from the urine of untreated humans and animals. The isolation of such substances is not easy. They may be present in low concentration and may be too unstable to withstand the chemical processes involved in their isolation. Apart from the importance of the isolation and characterisation of what might be called "physiologically normal conjugates", attempts to assess the relative amounts of conjugation with sulphuric and glucuronic acids in untreated animals may be misleading if based on results of experiments involving the administration of substances to the animals. This is the case with phenol where the relative amounts of phenyl glucuronide or phenyl sulphate formed and excreted appears to depend on the dose of phenol administered (Williams (1938)).

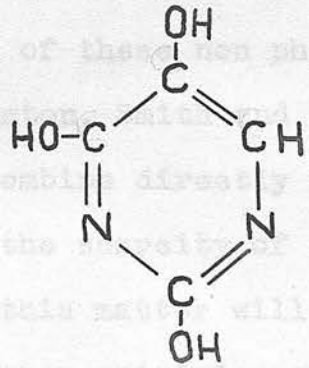
Baumann's discovery (1876 (a)) that the alkaline salts of phenyl sulphuric acid are almost non toxic was no doubt the original observation

supporting the view that conjugation with sulphuric acid is an important mechanism of "detoxication" by which the body decreased the toxicity and increased the solubility of certain substances prior to their elimination in the urine. This detoxication theory has been and to a certain extent still is widely held. As Marrian (1946) has pointed out however, in connection with certain steroids conjugated with glucuronic acid, it seems unlikely that this process of conjugation is merely one of detoxication. Especially in the case of steroid hormones and their metabolites it may be a normal metabolic process, possibly concerned with the transport of the steroids within the body.

There has been much controversy regarding the source of sulphate for conjugation with phenols. Reviewing the question Williams (1947) states that, "the balance of evidence indicates that ethereal sulphates are formed by the direct union of the sulphate ion with the phenol". The sulphate may be of endogenous origin, from the break down of protein, or exogenous in the form of administered sulphate.

Considering the nature of the hydroxyl groups entering into conjugation with sulphuric acid Williams and his co workers (Dodgson et al (1948))

have suggested that ethereal sulphate formation is related to the ionization of phenolic hydroxyl groups. They refer to the extremely low ionization constants of alcoholic hydroxyls compared with phenols. A survey of phenols known to conjugate with sulphate showed that the pK's of the OH groups varied from about 7 to 10 (Anderton, Smith and Williams (1948)). A second factor for ethereal sulphate formation was suggested by the observation that, of a number of pyrimidines possessing pK's of the same order as phenols, veronal, uracil, alloxan and isobarbituric acid, only the last named formed an ethereal sulphate. The formula of the barbituric acid may be written as a heterocyclic phenol thus



This structure possesses the enolic system C=C(OH)-C not found in the other pyrimidines. Williams thus suggests that the two conditions for sulphate conjugation are (a) pK within the range 7 to 10 and (b) the OH in the enolic system C=C(OH)-C. Ascorbic

acid forms no ethereal sulphate because although it fulfills condition (b) the pKs of the two hydroxyls concerned both lie outside the range 7 to 10.

These theories take no account of the fact that ethereal sulphate conjugates involving alcoholic hydroxyl groups are found in the animal organism. The chondroitin sulphate of gastric mucin may belong to a special class but urinary ethereal sulphates of non phenolic steroids are known. Reference has already been made to sulphates of androsterone, dehydroepiandrosterone, Δ^{16}allopregnen-3β-ol-20-one and allopregnane-3β-ol-20-one.

Since it has not been demonstrated that ethereal sulphate formation takes place after the administration of these non phenolic steroids, Williams suggests (Anderton, Smith and Williams (1948)) that they may not combine directly with sulphate in vivo. On account of the scarcity of these steroids it is unlikely that this matter will be readily settled.

Another criticism of Williams' views is that they will not explain the failure of certain phenols to form ethereal sulphates. Thus for instance catechol sulphates have not been found in nature, but on feeding catechol to rabbits Williams found an increase in ethereal sulphate (Garton and Williams (1948)).

He attributes this to the formation of a catechol monosulphate, although he was unable to isolate the substance. On theoretical grounds he considers that the formation of a disulphuric acid ester from the mono ester is unlikely, since catechol monosulphate is a phenol with a strongly acid group in the ortho position. This structure appears to inhibit the reactivity of the second hydroxyl. On physico-chemical grounds (Pauling (1945)) the reactivity of one hydroxyl in catechol may be suppressed by hydrogen bonding. A similar explanation is offered for the failure to find the ethereal sulphate of salicylic acid in the urine of rabbits receiving salicylic acid.

Hydrogen bonding does not prevent the formation of catechol mono and disulphates in vitro. Thus Baumann (1878) prepared catechol mono and disulphates synthetically. It is also interesting to note that Baumann and Herter (1877) claim to have isolated salicylic acid ethereal sulphate from the urine of dogs.

There would appear to be no satisfactory explanation for the failure to obtain catechol sulphates from urine.

It is interesting to note that the only known conjugates of the phenolic oestrogens are the sulphate of oestrone and the glucuronide of oestriol, although these substances oestrone and oestriol have

very similar dissociation constants (oestrone pK 9.96, oestriol pK 9.21). The recent work of Cohen and Bates (1949) has however suggested the possible existence of oestriol sulphate, although this substance has not actually been isolated.

In favour of his views that the only hydroxy compounds which form ethereal sulphates on administration to animals are phenols, Williams (Anderton et al. (1950)) suggests that it is significant that the enzyme phenolsulphatase only hydrolyses phenolic ethereal sulphates but not alcoholic sulphates. The present author is of the opinion that a thorough reinvestigation of the specificity of sulphatase preparations is called for. In particular it would be of interest to check the effect of so called "phenolsulphatase" on non phenolic steroid sulphates.

2. p-ethylphenol in the excretions and secretions of vertebrates.

Although it was suggested by Baumann in 1879 that p-ethylphenol might be formed in the animal body by the degradation of tyrosine, the presence of this substance was not reported until 1927 when Walbaum and Rosenthal (1927) and Pfau (1927) isolated it from the dried scent glands of the beaver. More recently, Lederer (1943, 1946) isolated p-ethylphenol from an extract of acid-hydrolysed pregnant mare urine by careful fractional distillation of the phenolic non ketonic portions. The p-ethylphenol was identified as the phenyl urethane and glycollic acid ether. Lederer believes this phenol to be derived from tyrosine, and that it is a normal constituent of animal urine.

In the present work the isolation of p-ethylphenyl sulphuric acid from urine as the potassium salt is reported for the first time. This substance was obtained initially from the urine of an ovariectonised goat receiving large doses of progesterone and hexoestrol. Subsequently it was also isolated from the urine of a normal goat.

In view of the belief (Williams 1947 (b)) that p-cresol is quantitatively the most important phenol in the urine of vertebrates, it is noteworthy that no clear cut evidence has been obtained for the presence of p-cresyl sulphuric acid in the urine

examined. The fact that the derivatives of the p-ethylphenol, prepared from the hydrolysis products of the sulphate, required frequent recrystallisation before constant melting points could be obtained, might suggest that the isolated substance was contaminated with appreciable amounts of the sulphate of p-cresol or of other phenols. Nevertheless the present work indicates that in the goat p-ethyl phenyl sulphuric acid is excreted in larger amount than p-cresyl sulphuric acid. Lederer (1946) found that p-ethylphenol amounted to only 5 to 13% of the p-cresol present in pregnant mare's urine. On the other hand Lederer and Polonsky (1942) found various phenols, but no p-cresol in the dried scent glands of the beaver, an animal which appears to deposit in the scent glands substances which other vertebrates would normally excrete in the urine after conjugation.

Lederer (1949) reviewing the biochemistry of some mammalian secretions and excretions considers that a definite species difference exists between the phenols excreted by certain vertebrates, referring to the pregnant mare, beaver and goat excretions (Grant (1948)). It appears to the author however that further investigation will be necessary before this claim can be substantiated. The

animals compared should be in the same physiological state. It seems unwise to compare the pregnant mare with the normal goat.. Furthermore the total phenols in the urine must be compared. It is well known from the work of Folin and Denis (1915) that a large amount may occur free in addition to being conjugated with glucuronic or sulphuric acid. Although p-ethyl phenyl sulphuric acid appears to be excreted in larger amounts than p-cresyl sulphuric acid, no evidence has been obtained regarding the relative amounts of <u>total</u> phenols excreted by the goat.

<u>3. The Isolation of p-ethylphenyl sulphuric acid as potassium salt from goat urine. (Treated Goats).</u>

The experimental animals have been described on page 119. Goats were prevented from chewing wooden parts of metabolism cages which might have contributed phenols to their diet.

3.5 l. urine collected during 7 days from a goat receiving treatment were extracted with butanol as described on page 120 The residue from the washed butanol extract was dissolved in 500ml. water, chilled to 0°, acidified to pH2 with HCl and rapidly extracted

four times with 200 ml. volumes of chilled ether. The ether washed aqueous solution was made alkaline to litmus with NaOH and extracted four times with 200 ml. volumes of butanol. After washing twice with 50 ml. volumes of water the butanol was evaporated under reduced pressure to give 5 g. of light brown solid.

The above-described solid was dissolved in a minimum of water, and an equal volume of saturated potassium acetate solution added. The crystalline precipitate formed was washed twice with half-saturated potassium acetate solution, and dissolved in 200 ml. butanol. After washing several times with water, the butanol was evaporated in vacuo, leaving 1.933 g. of nearly white crystals. After crystallization from 4% (v/v) aqueous acetone, crystals melting at 264-266° were obtained.

The amounts of potassium p-ethylphenyl sulphate, isolated in the pure state from the urine of the goat receiving progesterone and hexoestrol, are shown in Table 5.

TABLE 5.

Potassium p-ethylphenyl sulphate from goat urine.

Batch.	Collection dates.	Volume.	Wt. potassium p-ethyl-phenyl sulphate.
1.	20th-26th Jan. 1947.	3.5 l.	1.9330 g.
2.	27th-31st.	2.6 "	2.0027 "
3.	3rd - 8th Feb.	3.5 "	0.3195 "
4.	24th-28th	3.5 "	0.2610 "

The fact that batches 1 and 2 were worked up without delay whereas 3 and 4 were dealt with after several weeks' storage of extracts at 0°C., may account for the decrease in yield.

The material thus obtained dissolved readily in water, and gave a test for sulphate after warming with HCl. Millon's reagent gave a positive reaction after heating several minutes at 100°. The Tollens' reaction for glucuronic acid was negative. N, P and halogens were absent.

After drying several days in vacuo over P_2O_5 the following analytical results were obtained: C, 40.05, 40.09; H, 3.86, 3.79; S, 14.0, 13.9; K, 16.5, 16.8; potassium ethylphenyl sulphate ($C_8H_9SO_4K$) requires C, 39.98; H, 3.78; S, 13.34; K, 16.27%.

0.154 g. of the above-described potassium salt, and 0.225 g. of p-toluidine hydrochloride were dissolved separately in 5 ml. lots of water. The

two solutions were warmed to 50° and mixed. The precipitate of p-toluidine salt formed was recrystallized three times from water at 50°, giving a product melting at 164-166° (decomp.). Mixed with a sample of authentic p-toluidine salt of p-ethylphenyl sulphuric acid, there was no depression in melting point. Found: C, 58.25, 58.24; H, 6.02, 6.02; p-toluidine salt of p-ethylphenyl sulphuric acid ($C_{15}H_{19}NO_4S$) requires C, 58.24; H, 6.19. Heating of the p-toluidine salt in aqueous solution above 50° causes marked decomposition.

4. Hydrolysis of the potassium salt of the isolated sulphate.

Hydrolysis was effected by boiling the potassium salt with approximately N-HCl for 20 min., cooling, and extracting with ether. The residue obtained on evaporation of the washed ether was distilled at 100° and 0.1 mm. to give a white crystalline solid. This had a strong phenolic smell and gave a blue colour, turning to dirty green, with ferric chloride solution.

Benzoylation by the Schotten-Baumann method yielded a product which, after six recrystall-

izations from moist ethanol, melted at 59-60°. Mixed with authentic p-ethylphenyl benzoate (m.p.60°) the m.p. was 58-60°. Mixed with authentic p-cresyl benzoate (m.p.71°) the m.p. was 53°. Found: C, 79.64, 79.49; H, 6.06, 6.05; Calc. for p-ethylphenyl benzoate ($C_{15}H_{14}O_2$): C, 79.64; H, 6.23, for p-cresyl benzoate ($C_{14}H_{12}O_2$): C, 79.23; H, 5.7%.

The oxyacetic acid derivative of the ether-soluble hydrolysis product, after five recrystallizations, melted at 94-95°. Mixed with authentic p-ethylphenoxyacetic acid (m.p. 95-96°) the m.p. was 94-95° and mixed with authentic p-methylphenoxyacetic acid (m.p. 136°) the m.p. was 97-98°). 5.106 mg. dissolved in water required 1.17 ml. 0.0236N-NaOH giving an equivalent of 184.9. Calc. for p-ethylphenoxyacetic acid 180.2.

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5. Isolation of p-ethylphenyl sulphuric acid from normal goat urine as potassium salt.

By the processes described above 5.4 l. normal goat urine gave 1.15 g. potassium salt. The p-toluidine salt prepared from this gave C, 58.46, 58.12; H, 6.11, 5.96. p-Toluidine salt of p-ethylphenyl sulphuric acid ($C_{15}H_{19}NO_4S$) requires C, 58.24

H, 6.19%. The benzoate obtained from the ether-soluble hydrolysis product, after six recrystallizations from ethanol, melted at 57°. Mixed with authentic p-ethylphenyl benzoate (m.p. 60°) the m.p. was 56-57°. After seven recrystallizations from water the oxyacetic acid melted at 95°; mixed with authentic p-ethylphenoxyacetic acid the m.p. was 94-95°.

SUMMARY.

PART I.

1. It has been shown that rat and rabbit livers contain an enzyme system capable of destroying pregnanediol. The destruction is inhibited by the absence of oxygen, to some extent by azide, but not at all by cyanide. Some destruction takes place in nitrogen if methylene blue is added. The formation of conjugates will not account for the pregnanediol which disappears.

2. In a single experiment with NaPG no destruction of pregnanediol occurred if saccharate was employed to inhibit the hydrolysis of the glucuronide.

3. In a large scale experiment a very small amount of pregnane-3α-ol-20-one was isolated after incubation of pregnanediol with a liver powder. This result may be of doubtful value on account of the low yield.

4. It is not yet known whether these observations are of any biochemical significance.

PART II.

1. A modified method for the isolation and purification of HOG and NaOG from human pregnancy urine is described.

2. The sodium salt (NaOG) has been hydrolysed by ox spleen β glucuronidase, and the uronic acid so liberated has been oxidized to D-glucosaccharic acid which has been identified as its dibenziminazole derivative. It is therefore probable that the uronic acid moiety is D-glucuronic acid.

3. Results of a preliminary study of the oestriol moiety obtained from the products of enzymic hydrolysis of NaOG would appear to justify further investigation of the possibility that other substances closely related to oestriol are present.

4. No progress has been made with attempts to determine the position of attachment of the glucuronic acid to the oestriol moiety. It has not been found possible to prepare a fully methylated oestriol glucuronidic acid, nor to prepare oestriol trimethyl ether. The preparation and properties of a number of derivatives are described for the first time.

PARTS III & IV.

1. The examination of urine from goats receiving large amounts of progesterone failed to show the presence of pregnanediol, or other related steroids.

2. In the course of this work p-ethylphenyl sulphuric acid was isolated from the urine of progesterone treated goats. It was subsequently obtained from the urine of a normal goat. This compound, which appears to be quantitatively the most important phenol sulphate present in goat urine, had not previously been obtained from animal sources.

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 PRINTED IN GREAT BRITAIN

p-Ethylphenylsulphuric Acid in Goat Urine

By J. K. GRANT, *Department of Biochemistry, University of Edinburgh*

(*Received* 13 *April* 1948)

Although it was suggested by Baumann in 1879 that *p*-ethylphenol might be formed in the animal body by the degradation of tyrosine, the presence of this substance was not reported until 1927, when Walbaum & Rosenthal (1927) and Pfau (1927), isolated it from the dried scent glands of the beaver. More recently, Lederer (1943, 1946) isolated *p*-ethylphenol from an extract of acid-hydrolyzed pregnant mare urine.

In the present work the isolation of *p*-ethylphenylsulphuric acid from urine as the potassium salt is reported for the first time. This substance was initially obtained from the urine of an ovariectomized goat, which had received large doses of progesterone and hexoestrol. Subsequently it was also isolated from the urine of a normal goat.

In view of the belief (Williams, 1947) that *p*-cresol is quantitatively the most important phenol in the urine of vertebrates, it is noteworthy that no clear-cut evidence has been obtained for the presence of *p*-cresyl sulphuric acid in the urine examined. The fact that the derivatives of *p*-ethylphenol, prepared from the hydrolysis product of the sulphate, required frequent recrystallization before constant melting points could be obtained, might suggest that the isolated substance was contaminated with appreciable amounts of the sulphate of *p*-cresol or of other phenols. Nevertheless, the present work indicates that in the goat *p*-ethylphenylsulphuric acid is excreted in larger amount than *p*-cresylsulphuric acid.

EXPERIMENTAL

The experimental animals were mature ovariectomized goats on a diet of hay and a mixture of bran, linseed cake, oats and beans. For the purpose of lactation studies under the direction of Dr S. J. Folley at the National Institute for Research in Dairying, they received 1 mg. hexoestrol and 40 mg. progesterone daily. Dr Folley and Mr A. T. Cowie kindly arranged for the collection of urine, and for its dispatch to this laboratory.

The urine was collected quantitatively under butanol. Daily collections were stored in the refrigerator. The pooled 7-day collections received were worked up for conjugated substances. Normal goat urine was collected similarly from an animal receiving no treatment.

Extraction of urine

Urine (3·5 l.) from a treated goat was acidified to pH 3 with HCl, and rapidly extracted with butanol at low temperatures. The butanol extract was washed with N/3-NaOH, and water, and evaporated to dryness under reduced pressure. The residue was dissolved in water, chilled to 0°,

acidified to pH 2 with HCl, and rapidly extracted with chilled ether. The ether-washed aqueous phase was made alkaline to litmus with NaOH, and extracted with butanol. After washing with water the butanol was evaporated *in vacuo*, leaving 5 g. of light brown solid.

Preparation of potassium and p-*toluidine salts*

The above-described solid was dissolved in a minimum of water, and an equal volume of saturated potassium acetate solution added. The crystalline precipitate formed was washed twice with half-saturated potassium acetate solution, and dissolved in 200 ml. butanol. After washing several times with water, the butanol was evaporated *in vacuo*, leaving 1·933 g. of nearly white crystals. After crystallization from 4% (v/v) aqueous acetone, crystals melting at 264–266° (corr.) were obtained.

The material thus obtained dissolved readily in water, and gave a test for sulphate after warming with HCl. Millon's reagent gave a positive reaction after heating several minutes at 100°. The Tollens' reaction for glucuronic acid was negative. N, P and halogens were absent.

After drying for several days *in vacuo* over P_2O_5 the following analytical results were obtained: C, 40·05, 40·09; H, 3·86, 3·79; S, 14·0, 13·9; K, 16·5, 16·8; *potassium ethylphenylsulphate* ($C_8H_9SO_4K$) requires C, 39·98; H, 3·78; S, 13·34; K, 16·27%.

0·154 g. of the above-described potassium salt, and 0·225 g. of *p*-toluidine hydrochloride were dissolved separately in 5 ml. lots of water. The two solutions were warmed to 50° and mixed. The precipitate of *p*-toluidine salt formed was recrystallized three times from water at 50°, giving a product melting at 164–166° (corr.) (decomp.). Mixed with a sample of authentic *p*-toluidine salt of *p*-ethylphenylsulphuric acid, there was no depression in melting point. Found: C, 58·25, 58·24; H, 6·02, 6·02. p-*Toluidine salt of* p-*ethylphenylsulphuric acid* ($C_{15}H_{19}NO_4S$) requires C, 58·24; H, 6·19. Heating above 50° caused marked decomposition of the *p*-toluidine salt of the sulphate.

Hydrolysis of the potassium salt

Hydrolysis was effected by boiling the potassium salt with approximately N-HCl for 20 min., cooling, and extracting with ether. The residue obtained on evaporation of the washed ether was distilled at 100° and 0·1 mm. to give a white crystalline solid. This had a strong phenolic smell and gave a blue colour, turning to dirty green, with ferric chloride solution.

Benzoylation by the Schotten-Baumann method yielded a product which, after six recrystallizations from moist ethanol, melted at 59–60° (corr.). Mixed with authentic *p*-ethylphenyl benzoate (m.p. 60°, corr.) the m.p. was 58–60° (corr.). Mixed with authentic *p*-cresyl benzoate (m.p. 71°, corr.) the m.p. was 53°. Found: C, 79·64, 79·49; H, 6·06, 6·05. Calc. for *p*-ethylphenyl benzoate ($C_{15}H_{14}O_2$): C, 79·64; H, 6·23, for *p*-cresyl benzoate ($C_{14}H_{12}O_2$): C, 79·23; H, 5·70%.

The oxyacetic acid derivative of the ether-soluble hydrolysis product, after five recrystallizations, melted at 94–95° (corr.). Mixed with authentic *p*-ethylphenoxyacetic acid (m.p. 95–96°, corr.) the m.p. was 94–95° (corr.) and mixed with authentic *p*-methylphenoxyacetic acid (m.p. 136°, corr.) the m.p. was 97–98° (corr.). 5·106 mg. dissolved in water required 1·17 ml. 0·0236 N-NaOH giving an equivalent of 184·9. Calc. for *p*-ethylphenoxyacetic acid 180·2.

Normal goat urine

By the processes described above 5·4 l. normal goat urine gave 1·15 g. potassium salt. The *p*-toluidine salt prepared from this gave C, 58·46, 58·12; H, 6·11, 5·96. p-*Toluidine salt of* p-*ethylphenyl sulphuric acid* ($C_{15}H_{19}NO_4S$) requires C, 58·24; H, 6·19%. The benzoate obtained from the ether-soluble hydrolysis product, after six recrystallizations from ethanol, melted at 57° (corr.). Mixed with authentic *p*-ethylphenyl benzoate (m.p. 60°, corr.) the m.p. was 56–57° (corr.). After seven recrystallizations from water the oxyacetic acid melted at 95°; mixed with authentic *p*-ethylphenoxyacetic acid the m.p. was 94–95°.

SUMMARY

1. The isolation of *p*-ethylphenylsulphuric acid from animal sources, as the potassium salt, is reported for the first time.

2. The *p*-ethylphenylsulphuric acid was obtained from goat urine, in which it appears to be quantitatively the most important phenylsulphuric acid.

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