# XII. THE ISOLATION AND IDENTIFICATION OF A COMBINED FORM OF OESTRIOL IN HUMAN PREGNANCY URINE.

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In the past scant attention has been paid to the problem of the chemical nature of the "combined" ether-insoluble forms of the oestrogenic substances which are present in human and equine pregnancy urines and which yield the free hormones by acid hydrolysis. Two years ago the present authors planned a research which, it was hoped, would culminate in the isolation and chemical identification of these compounds. At the outset it was realised that slow progress would be made if only biological methods of assaying oestrogenic potency were available. Efforts were, therefore, first directed towards devising a convenient and accurate chemical method of assay. Fortunately, Kober [1931] had previously described a colour reaction for oestrin which, after the introduction of certain modifications, was found suitable for the purpose. Using such a modified Kober test, a method of purification and fractionation of pregnancy urine extracts was elaborated which allowed separate determinations of oestrone and oestriol to be made with a reasonable degree of accuracy [Cohen and Marrian, 1934]. A study was then made [Cohen and Marrian, 1935] of the factors influencing the hydrolysis of the combined forms of the two hormones. This made possible the accurate assay of the oestrone and oestriol present in human pregnancy urine and also provided evidence regarding the stability of the compounds so that methods for their purification could be more readily devised.

In the present communication are reported the results of attempts made to isolate and identify the combined form of oestriol present in human pregnancy urine. Since the amounts of oestrone present in human urine are relatively so small, it was felt that an attempt to isolate the combined form of this compound from the same source would at present be unprofitable.

## *Methods of concentration.*

In 1932 experiments (unpublished) were carried out by one of us (G. F. M.) in collaboration with Mr E. R. Smith which suggested that the "combined" oestrin could be extracted from evaporated urine residues by ethyl alcohol or from untreated urine by butyl alcohol. Owing to the difficulties of biological assay the results were not entirely conclusive and the work was not continued at that time. In the present work it has been found that the combined oestrone and oestriol can be almost quantitatively extracted from urine by both these methods. For the initial process extraction with butyl alcohol was preferred since it eliminated the time-consuming necessity of evaporating the urine to dryness.

In order to elaborate methods of further purification of the butyl alcohol extracts, it was found helpful to make an assumption about the probable nature of the oestriol complex. It seemed reasonable to suppose that the large water-insoluble oestriol molecule would be held in aqueous solution in the urine by

combination as an ester with some polybasic acid. Although subsequent work failed to prove the correctness of this hypothesis, its temporary adoption was justified since it enabled a satisfactory method of isolation to be devised.

Assuming the acidic nature of the complex, it was thought desirable to acidify the urine slightly before extraction with butyl alcohol, since it seemed probable that salts of the complex would be less soluble in butyl alcohol than the free acid. This procedure involves no risk of loss of the complex since it was previously shown [Cohen and Marrian, 1935] that little hydrolysis of combined oestrin occurs even at $p_H$ 1·0 at low temperatures. The belief that the complex possessed a free acidic group was confirmed by the discovery that it could be removed from the butyl alcohol by extraction with alkaline solutions, preferably $N/10$ sodium hydroxide. By acidification of such alkaline extracts, extraction with butyl alcohol and evaporation of the latter, concentrates containing most of the combined oestriol originally present in the urine and almost free from urea were obtained. Extraction of such concentrates with 90% ethyl alcohol removed most of the complex leaving behind considerable quantities of inorganic salts.

The evidently acidic nature of the oestriol complex suggested the use of an organic base such as pyridine as a possible means of further purification. It was found that whilst dry pyridine would not readily remove the complex from the product obtained by the evaporation of the 90% alcoholic extract, pyridine containing small amounts of water was extremely effective; 92% aqueous pyridine proved to be the most suitable mixture for the purpose. This pyridine-soluble material was found by colorimetric assay to contain about 0·5–0·6% of oestriol.

Advantage was now taken of the low solubility of the oestriol complex in anhydrous pyridine for devising a method of removing the material from the pyridine solution, thus obviating the tiresome necessity of low temperature evaporation of the pyridine extract. The addition of a suitable amount of benzene to the pyridine solution caused an aqueous fraction to separate, and when this mixture was shaken with water the oestriol complex was quantitatively transferred to the aqueous phase. At the same time a further purification was effected in so far as about 25% of the original solids, including all the free oestriol, were left in the pyridine-benzene layer.

Extraction of the aqueous solution of the complex with a water-immiscible nitrogenous base now suggested itself. It was found that the complex could be readily removed from the aqueous extract by shaking with quinoline. Owing to the difficulty in evaporating the quinoline no data concerning the degree of purification effected were obtained, but since the aqueous phase remained highly pigmented, it was clear that some impurities were being removed. For the removal of the oestriol complex from the quinoline solution a process similar to that used in the pyridine process was impracticable since the solubility of the complex in quinoline is apparently independent of the presence of water. Attempts were made to remove the quinoline as its water-soluble hydrochloride by adding the extract to a large volume of butyl alcohol and washing the mixture with dilute hydrochloric acid. This process, however, resulted in a considerable loss of the oestriol complex and was therefore abandoned. Extraction of the quinoline with aqueous alkalis fortunately proved to be more effective. By shaking the quinoline solution with 10% aqueous sodium carbonate traces only of the complex but much pigmented material were transferred to the aqueous phase. On the other hand, extraction with $N/10$ sodium hydroxide removed over 80% of the complex. The procedure was therefore finally adopted

of extracting the complex from the quinoline with $N/10$ sodium hydroxide after preliminary washing with 10% sodium carbonate. The complex was separated from inorganic matter introduced during this purification by slight acidification of the alkaline extract and subsequent extraction with butyl alcohol. Assays on this alcoholic extract showed that the whole quinoline process had resulted in a 10-fold degree of purification with only 30% loss of the total combined oestriol obtained from the preceding pyridine process.

The success attending extraction of the quinoline solution with aqueous solutions of varying degrees of alkalinity, suggested that it might be profitable to explore more fully the possibilities of a similar alkali fractionation of butyl alcohol solutions. A series of experiments conducted towards this end resulted in the adoption of the following process. The butyl alcoholic solution of the material obtained from the quinoline process was first washed with 0·8% sodium carbonate solution, thereby effecting the removal of much pigmented material but of only small amounts of the oestriol complex. The complex was subsequently removed from the butyl alcohol by extraction with $N/50$ sodium hydroxide. Inorganic material introduced by this process was separated in the usual manner by transference of the combined oestriol to butyl alcohol. This process resulted in about a 4-fold purification with only 12% loss in the total combined oestriol. At this stage the product contained about 15%[1] of oestriol.

In an attempt to devise methods of further purification the solubilities of this material in various organic solvents were studied. Cold acetone extracted the combined oestriol quantitatively, leaving behind a brown oil which amounted to about 20% by weight of the original material. Attempts to purify further the acetone-soluble material by the addition of solvents such as benzene and chloroform, in which the complex is insoluble, were ineffective.

It was thought that at this stage it might be profitable to attempt the separation and purification of a metallic salt of the complex. Alcoholic potassium hydroxide was found to precipitate a heavy greyish-white material from an alcoholic solution of the acetone-soluble fraction. This precipitate contained about 85% of the combined oestriol present. Since the supernatant liquid was deeply pigmented, it was clear that a considerable degree of purification had been effected.

On adding dilute aqueous barium chloride to an aqueous solution of the crude potassium salt, a light yellow precipitate was thrown down. The supernatant liquid was again deeply pigmented and was found to contain little combined oestriol. On treatment of the barium salt with hot dilute hydrochloric acid and filtering while hot, a solution was obtained which on cooling deposited a nearly white amorphous solid. A second precipitation from hot water yielded a material which contained about 50% by weight of oestriol.

*Nature of the final product.*

This material, although not crystalline, contained such a high proportion of oestriol that it obviously represented a highly concentrated preparation of the sought-for complex. The substance melted at 193–197° with decomposition after preliminary sintering at 180°. Only 46 mg. of this final white precipitate being available it was decided to investigate its properties and chemical nature without any further attempts at purification.

[1] As later pointed out the method of oestrin assay employed on early concentrates gave values which, although relative, could not be regarded as strictly quantitative. They did, however, supply a working basis and hence are reported, even though subsequent work showed them to be 25–50% too low.

Sulphur, halogens, phosphorus and nitrogen were absent. Since many phenolic substances are excreted from the body in conjugation with glucuronic acid, the possibility was entertained that the complex might be a derivative of this acid. The fact that it decomposed at its melting-point with the evolution of gas lent some support to this idea. As the amounts of material were so small it was not considered advisable to try to isolate glucuronic acid or one of its easily identifiable derivatives from the products of acid hydrolysis of the complex. Since, however, the Tollens naphthoresorcinol test was strongly positive on as little as 0·5 mg. it seemed very probable that the material was indeed a glucuronic acid derivative.

Carbon and hydrogen determinations gave figures closely in agreement with those required for a substance of the formula $C_{24}H_{34}O_9$. An oestriol glucuronic acid complex would have the formula $C_{24}H_{32}O_9$; there can, therefore, be no reasonable doubt concerning the nature of the isolated substance. Further evidence was obtained when the barium salt of the complex was found to have a barium content close to that required for the barium salt of an oestriol glucuronic acid.

The substance did not reduce Benedict's solution indicating that the conjugation is through the terminal aldehyde group of the glucuronic acid. After hydrolysis with dilute hydrochloric acid the reduction test was strongly positive. Millon's test was strongly positive in the cold, which may perhaps indicate that the phenolic hydroxyl group of the oestriol is free. The possibility was borne in mind that the nitric acid present in the reagent might have caused sufficient hydrolysis to render the test positive even though the phenolic group was originally masked in the compound. The fact that salicin, under the same conditions, gave a completely negative Millon test lent no support to this idea.

The questions of the physiological activity of this interesting derivative of oestriol and of its possible relationship to the placental principle "emmenin" [*cf.* Collip *et al.*, 1934] will be dealt with in subsequent communications.

## Experimental.

### *Method of assay.*

The amounts of combined oestriol present in the concentrates at different stages of the purification process were determined colorimetrically by the method previously described [Cohen and Marrian, 1934]. The conditions of acid hydrolysis previously found to be satisfactory for fresh urine [Cohen and Marrian, 1935], however, had to be altered owing to the relative or complete absence of urea from the concentrates. In general the following procedure was finally adopted: an aliquot portion of the concentrate, estimated roughly to contain about 1 mg. of oestriol, was diluted to 100 ml. with water, acidified with HCl to $p_H$ 1·0 and, after the addition of a further 0·5 ml. of concentrated acid, heated in the autoclave at 15 lb. pressure for 1½ hours. The free oestriol in the hydrolysis mixture was estimated in the usual manner.

Although no great confidence was felt that this method of hydrolysis gave a quantitative yield of oestriol from its combined form for all urine concentrates, the results were of comparative value and were sufficiently accurate to make possible the elaboration of a method of purification. As will be shown later, it was found possible to carry out direct colorimetric assays without previous hydrolysis on certain of the more highly purified concentrates obtained in the later stages of the process.

*Collection and extraction of urine.*

100 l. of fresh urine, from women in the 8th and 9th months of pregnancy, were collected in bottles containing toluene as a preservative and evaporated in a vacuum still to about one-fifth of the original volume. When it was not possible to concentrate the urine immediately after collection, it was stored at 0° in order to minimise bacterial hydrolysis of the combined oestriol. The concentrate was acidified to $p_H$ 2·0 with HCl, saturated with NaCl and then extracted six times with butyl alcohol, the total volume of the latter used being about equal to that of the concentrated urine. The saturation with NaCl facilitated the breaking of the troublesome emulsions and furthermore by lowering the solubility of the butyl alcohol in the aqueous phase effected a considerable economy in the quantity of the solvent used.

*Extraction of the combined oestriol from butyl alcohol by aqueous alkalis.*

The following preliminary experiment was carried out. Four 100 ml. portions of the butyl alcoholic extract of a urine concentrate, each containing 1·3 mg. of oestriol in the combined form (by colorimetric assay), were extracted twice with 50 ml. volumes of (*a*) 10 % $Na_2CO_3$, (*b*) 25 % $Na_2CO_3$, (*c*) *N*/10 NaOH and (*d*) *N* NaOH respectively. The alkaline extracts were each assayed for total oestriol after acid hydrolysis and were shown to contain (*a*) 0·750 mg., (*b*) 0·325 mg., (*c*) 1·250 mg. and (*d*) 1·280 mg. of oestriol respectively.

The butyl alcoholic extract after careful neutralisation was therefore extracted six times with *N*/10 NaOH, the total volume of the latter equalling that of the original extract. Approximately 75 % of the total oestriol present in the butyl alcohol was thus removed. In order to obtain a residue concentrate the alkali extract was acidified to $p_H$ 2·0 with HCl, saturated with NaCl and extracted six times with butyl alcohol. This extract was washed with a small volume of saturated NaCl (acidified to $p_H$ 2·0) to remove traces of urea that might have come through, then made slightly alkaline,[1] and evaporated to dryness under reduced pressure.

*Extraction of the combined oestriol by aqueous alcohol.*

Preliminary experiments showed that from such concentrates, 97 % ethyl alcohol, slightly acidified to neutralise the free alkali in the concentrate, would only extract about 10 % of the combined oestriol present. On the other hand, acidified 90 % alcohol extracted over 80 % of this material. Unacidified 90 % alcohol extracted only about 40 % of the combined oestriol present, clearly demonstrating the lower solubility of the alkali salt of the complex. The butyl alcohol residue was therefore shaken vigorously four times with 90 % ethyl alcohol containing sufficient acid to bring the $p_H$ of the suspension to about 5·0. It was found advantageous to add some fine sand to the flask to facilitate the separation of the solid from the sides. A total volume of 90 % ethyl alcohol equal to about one-eighth of the volume of original concentrated urine was used. The alcoholic extracts were decanted from the insoluble solid, cleared by centrifuging, neutralised and evaporated to dryness under reduced pressure. Assays showed that only traces of oestriol remained in the alcohol-insoluble fraction.

[1] Wherever possible all solutions were made slightly alkaline before evaporation. As the stability of the complex appears to be greater at alkaline reactions losses due to slow hydrolysis have been minimised in this way.

*Extraction with pyridine.*

In order to determine the most effective pyridine solution for removing the oestriol complex from the 90% ethyl alcoholic residue three samples, each containing 1·20 mg. total oestriol, were extracted with pyridine solutions containing different proportions of water. Subsequent analysis showed that a 90% aqueous pyridine extract contained 1·15 mg., a 95% extract contained 0·85 mg. and a 98% extract contained 0·44 mg. of total oestriol. 92% aqueous pyridine was found to be just as effective for the purpose as the 90% solution and had the added advantage of removing less of the total solids.

The dry 90% ethyl alcoholic residue was therefore extracted six times with 92% aqueous pyridine, a total volume of the pyridine solution equal to one-eighth of the original volume of concentrated urine being used. The pyridine extracts were separated from the insoluble material by centrifuging and combined. Assays showed that about 16% of the total oestriol remained in the pyridine-insoluble material. Evaporation of an aliquot portion of the pyridine extract and colorimetric assay of the residue after hydrolysis showed that the total weight of the pyridine-soluble material was 126 g. and that it contained 0·421 g. of total oestriol.

*Distribution of pyridine extract between benzene and water.*

To the pyridine extract were added two volumes of benzene and one of water. These proportions had been found optimum with respect to purifications effected, to completeness of removal of the combined oestriol and to minimising the amount of pyridine carried into the aqueous phase. After shaking, the aqueous layer was run off and washed once with benzene to remove final traces of pyridine. Subsequent assays showed it to contain about 75% of the total oestriol originally present. Only free oestriol remained in the benzene-pyridine phase.

*Distribution between quinoline and sodium carbonate and hydroxide solutions.*

The aqueous solution containing the oestriol complex obtained by the pyridine purification process was extracted twice with quinoline, the total volume of the latter being about half that of the aqueous solution. Colorimetric assay showed that only traces of oestriol remained in the aqueous phase.

In a preliminary experiment three equal portions of this quinoline extract, each containing 1·40 mg. of oestriol, were extracted once with an equal volume of (*a*) 10% $Na_2CO_3$, (*b*) *N*/10 NaOH and (*c*) *N* NaOH solutions. Colorimetric assays carried out on these alkali extracts showed that they contained 0·030 mg., 1·260 mg. and 1·390 mg. respectively.

The following procedure was therefore adopted for the main bulk of the quinoline extract. The quinoline was first washed twice with 10% $Na_2CO_3$ and then extracted twice with *N*/10 NaOH. In each case the total volumes of $Na_2CO_3$ and NaOH used were approximately equal to that of the quinoline solution. The combined oestriol was recovered from the NaOH solution by acidification to $p_H$ 5·0 and extraction with butyl alcohol. An aliquot portion of the butyl alcoholic extract was washed with dilute HCl and then with water, evaporated to dryness, weighed and assayed for total oestriol. In this way it was determined that the total butyl alcoholic extract contained 5·9 g. of solids, of which approximately 0·25 g. was oestriol in a combined form.

*Distribution between butyl alcohol and sodium carbonate and hydroxide solutions.*

Several equal portions of the butyl alcoholic extract, each containing 0·7 mg. of combined oestriol in 5 ml. were extracted once with 5 ml. of a dilute alkaline solution. Colorimetric assays after hydrolysis showed that whereas 1 % $Na_2CO_3$ extracted only 0·115 mg. of combined oestriol, $N/10$ NaOH removed 0·445 mg.

The remainder of the butyl alcoholic extract (containing about 200 mg. oestriol), after washing with dilute HCl to remove traces of quinoline and then with water, was further washed several times with a total equal volume of 0·8 % $Na_2CO_3$ and then extracted with $N/50$ NaOH. These concentrations of carbonate and hydroxide tended to minimise losses of the complex. Assay showed that 48 mg. of oestriol nearly all of which was in the free state remained in the butyl alcohol.

The combined oestriol was recovered from the NaOH in the usual manner by extraction with butyl alcohol after acidification to $p_H$ 5·0. The butyl alcoholic extract was washed with water and evaporated to dryness. This material weighed 1·17 g. and by colorimetric assay after hydrolysis was shown to contain 0·133 g. of combined oestriol. It was found that this material gave, without preliminary hydrolysis, apparently satisfactory direct colorimetric assays. Such an assay showed 0·300 g. of combined oestriol to be present. It would seem possible that either the hydrolysis was incomplete or that during the hydrolysis extensive destruction of the liberated oestriol occurred.

*Precipitation of the potassium salt.*

Extraction of this material with cold acetone left 0·22 g. of a brown oil which contained no oestriol. The residue obtained by evaporation of the acetone solution was dissolved in the smallest possible volume of ethyl alcohol and to this solution was added saturated ethyl alcoholic KOH until precipitation was complete. This precipitate was collected by centrifuging and washed once with a small volume of ethyl alcohol. Direct colorimetric assay showed that 50 mg. of combined oestriol were lost in the supernatant liquid and washing.

*Precipitation and decomposition of the barium salt.*

The crude washed potassium salt was dissolved in a small volume of hot water and to this was added $N/10$ $BaCl_2$ until precipitation was complete. After cooling, the barium salt was centrifuged off and washed first with a little cold water and then with ethyl alcohol. 12 mg. of oestriol were lost in the supernatant liquid and washings.

The crude barium salt was suspended in a small volume of hot water and treated with dilute HCl drop by drop until all but a few dark-coloured particles had gone into solution. The hot solution was filtered; the filtrate on cooling deposited a white amorphous precipitate. This material was collected by centrifuging and further purified by dissolving in hot water, filtering and cooling. The final product was dried *in vacuo* over calcium chloride and weighed 46·3 mg. It melted at 193–197° with evolution of gas after sintering at 180°.

By working over the mother-liquors from the potassium and barium salt precipitations, 33 mg. more of material melting at about 170° were obtained.

*Properties and chemical nature of the final product.*

The material was soluble in hot water but less soluble in cold. It was precipitated from cold aqueous solution by half-saturation with ammonium sulphate. It was soluble in acetone, alcohol and ethyl acetate, and insoluble in ether, benzene and chloroform.

Qualitative tests by fusion with sodium showed that nitrogen, sulphur and halogens were absent. After decomposition with hot sulphuric and nitric acids no positive Briggs test for phosphorus was obtained.

A strongly positive naphthoresorcinol test (Tollens) was given by 0·46 mg. suspended in 1 ml. of water. 0·6 mg. failed to cause any visible reduction of 0·5 ml. of Benedict's reagent. 4·1 mg. were hydrolysed by heating with 3 ml. of $N/10$ HCl for 2 hours in an autoclave at 15 lb. pressure. After neutralisation with sodium bicarbonate the solution strongly reduced Benedict's reagent. Millon's reaction was strongly positive in the cold.

(Found: C, 61·60, 62·09; H, 7·19, 7·44 %. $C_{24}H_{34}O_9$ requires C, 61·76; H, 7·35 %. $C_{24}H_{32}O_9$ (oestriolglucuronic acid) requires C, 62·03; H, 6·95 %.)

The samples for combustion were dried over phosphorus pentoxide at 80° *in vacuo* and lost 4·95 % and 5·43 % of their weights respectively.

Theoretical loss of weight for $C_{24}H_{32}O_9$, 1·5 $H_2O$ = 5·6 %.

*Barium salt.* 10 mg. of the product were dissolved in 10 ml. of hot water. An equal volume of $N/10$ $BaCl_2$ was added and the white precipitate which formed on cooling the mixture was centrifuged. The precipitate was heated with 10 ml. of hot 50 % ethyl alcohol and the solution filtered from a small amount of insoluble material and cooled. The amorphous precipitate was centrifuged, washed with 0·5 ml. of cold water and dried *in vacuo* over $CaCl_2$.

(Found: Ba, 11·2 %. $(C_{24}H_{31}O_9)_2$ Ba requires Ba, 12·9 %.)[1]

*Oestriol content.* Direct colorimetric assays on 0·0652, 0·0931 and 0·1304 mg. gave figures of 52 %, 53 % and 49 % oestriol respectively. Calculated for $C_{24}H_{32}O_9$, 1·5 $H_2O$, 58·6 % oestriol.

Six samples of 0·243 mg. of the product were hydrolysed for 0, ½, 1, 2, 3 and 4 hours respectively with 50 ml. of $N/10$ HCl saturated with nitrogen (to minimise loss by oxidative destruction) in the autoclave at 15 lb. pressure. Each solution was made alkaline with 10 ml. of 10 % $Na_2CO_3$ and extracted with ether. Colorimetric assays were carried out on the residues obtained by evaporation of the water-washed ethereal solutions in the usual manner. The results are shown in the following table:

| Time of hydrolysis hours | Oestriol determined mg. |
|---|---|
| 0·0 | 0·019 |
| 0·5 | 0·049 |
| 1·0 | 0·052 |
| 2·0 | 0·092 |
| 3·0 | 0·083 |
| 4·0 | 0·0815 |

The maximum hydrolysis of 2 hours gave a figure of 38 % as the oestriol content of the compound.

## Summary.

A method is described by which a water-soluble, ether-insoluble non-crystalline substance containing approximately 50 % by weight of oestriol may be isolated from human pregnancy urine. The substance gives a strong naphthoresorcinol test for glucuronic acid. Its elementary composition and the barium content of its barium salt are in fair agreement with those required for an oestriolglucuronic acid ($C_{24}H_{32}O_9$). Since it does not reduce Benedict's solution (but does so after hydrolysis) it is clear that the oestriol is linked to the glucuronic acid by a glucosidic linkage through the aldehyde group of the latter. Since the compound gives a strong Millon test in the cold, this linkage possibly does not involve the phenolic hydroxyl of the oestriol unit.

[1] The authors are grateful to Dr Helen Stantial for carrying out this determination.

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