The Isolation and Properties of the Monoglucuronides of Stilboestrol, Hexoestrol and Dienoestrol

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The monoglucuronides of stilboestrol, hexoestrol and dienoestrol were isolated from the urine of rabbits during metabolism experiments described previously (Wilder Smith & Williams, 1948). Mazur & Schorr (1942) described the glucuronide of stilboestrol which they obtained from rabbits treated with stilboestrol. As far as we are aware the glucuronides of hexoestrol and dienoestrol have not been isolated before.

METHODS

Urine collection and extraction. The urine was collected twice daily in the presence of toluene and stored at 0–3°. In the dienoestrol experiments a few mg. of hydroquinone were added daily to the collection vessels to assist stabilization.

After acidification to Congo red with dilute HCl the urine was extracted by hand three times with an equal volume of ether. Within limits, the larger the volume of ether used for each extraction, the less danger there was of the formation of intractable foams. The remaining urine was then extracted in a continuous ether extractor for 24 hr. and the combined extracts dried over anhydrous Na₂SO₄. After concentration to 20–60 ml. the ether solution was set aside in the refrigerator to crystallize.

In the method used by Mazur & Schorr (1942) the ether extract was not dried, but was extracted with saturated NaHCO₃ solution from which the sodium salt of the glucuronide crystallized. This method gave somewhat low yields with stilboestrol and dienoestrol and in the case of hexoestrol yielded no glucuronide at all (except on one occasion). The free glucuronide sometimes crystallized well directly from the ether extract when in sufficiently high concentration, but where difficulty was encountered with dienoestrol or stilboestrol the NaHCO₃ method was used. In cases where this expedient yielded no crystalline sodium salt, the bicarbonate solution was acidified to Congo red, extracted with ether, the ether removed and the residue taken up in a small amount of acetone. Addition of the acetone solution to saturated aqueous NaHCO₃ usually yielded the crystalline sodium salt. Solution of the crude sodium salts in water followed by acidification in the cold with dilute HCl precipitated the free glucuronides as a plastic mass. After washing with a small volume of water, and drying *in vacuo* over CaCl₂, the crude solid was dissolved in a little acetone and filtered. On adding excess dry ether to the filtrate a slight amorphous precipitate was formed which was filtered off. On keeping the filtrate at 0–3° the glucuronide slowly crystallized.

It was not found possible to remove a molecule of water of crystallization from the hexoestrol glucuronide even after drying for 6 hr. *in vacuo* at 110°. Dienoestrol and stilboestrol glucuronides also hold water of crystallization tenaciously, which is, however, almost completely removed under the above conditions.

Biological assay. The oestrogenic activity of the glucuronides was tested by subcutaneous and intravaginal administration to spayed rats. The technique used for the assay by subcutaneous injection has been described previously (Wilder Smith & Williams, 1948). The intravaginal assay was carried out by the application of two doses of 0.01 ml. of oily solution into the rat vagina 24 hr. apart; smears were taken during the two succeeding days. In both cases positive responses were indicated by vaginal smears consisting of cornified and/or epithelial cells only and dose response curves were constructed and the ED₅₀ calculated by the method of Fisher & Yates (1943).

RESULTS

Chemical properties

Stilboestrol monoglucuronide. Mazur & Schorr (1942) report the melting point, analysis and optical rotation of this substance. We have found very similar properties, which are given here for confirmation. In addition the physiological properties are reported.

After recrystallization of the white needles several times from dry ether containing a trace of acetone and drying 2 hr. *in vacuo* at 110°, white, semi-fused needles were obtained, softening 100–110°, melting with decomposition 174–176°. After longer drying periods some samples yielded a pale yellow syrup. On drying *in vacuo* (0.1 mm. Hg) at 110° for 4 hr. 10.7 mg. glucuronide lost 0.7 mg. (6.5%) with extensive sintering. Theoretical loss for 1H₂O, 3.9; for 2H₂O, 7.5%. On exposing the dried sample to the atmosphere for 48 hr. at room temperature, the glucuronide regained 0.3 mg. in weight. $[\alpha]_D^{24} = -51.6^\circ$ ($c = 1.60$ in absolute ethanol); $[\alpha]_D^{24} = -57.6^\circ$ ($c = 0.179$ in absolute ethanol). Equivalent weight = 451 (using 0.01 N-NaOH and methyl red as indicator). Theory = 444. Found: C, 64.1; H, 6.7. Calc. for C₂₄H₂₈O₈: C, 64.8; H, 6.4%.

Hexoestrol monoglucuronide. This was obtained in the crude state by direct crystallization from an

ether extract of the acidified urine. Difficulty was experienced in the purification of this substance since it did not crystallize as the sodium salt from saturated aqueous sodium bicarbonate solution, except in one experiment where the concentration of glucuronide used was higher than usual. Further crystallization either from acetone and benzene or from acetone and ether mixtures was sometimes difficult. It was therefore purified by dissolving in sodium bicarbonate solution, extracting with ether, making the aqueous layer acid to Congo red paper with dilute hydrochloric acid, extracting with ether and drying over anhydrous sodium sulphate. The ether was removed by distillation, and the residue

and insoluble in benzene and light petroleum and gave a positive Tollens test (after 30 min. acid hydrolysis). After drying 2 hr. *in vacuo* at 110° fine white needles were obtained, sintering at 105–115° and melting at 175–183° (with decomp.). On drying *in vacuo* (0.1 mm. Hg) at 110° for 4 hr. 9.9 mg. glucuronide lost 0.1 mg. (1.0 %) with little sintering. On exposing the dried sample to the atmosphere for 48 hr. at room temperature 0.1 mg. was regained in weight. $[\alpha]_D^{24} = -41.5^\circ$ ($c = 0.560$ in absolute ethanol). $[\alpha]_D^{24} = -47.2^\circ$ ($c = 0.182$ in absolute ethanol). Equivalent weight = 426. Theory for monoglucuronide = 442. Found: C, 64.4; H, 7.0. $C_{24}H_{26}O_8$ requires C, 64.8; H, 5.9 %.

Table 1. *Oestrogenic activity of stilboestrol, hexoestrol and dienioestrol monoglucuronides in rats*

(Figures for free oestrogens given for comparison in brackets.)

Oestrogen	ED ₅₀ (in μ g.) with 95 % fiducial limits	
	By subcutaneous injection in aqueous solution	By intravaginal application in oily solution
Stilboestrol monoglucuronide	5.8 (5.5–6.2)	0.04 (0.03–0.05)
[Stilboestrol]	0.35	0.003]
Hexoestrol monoglucuronide	11.5 (8.7–15.3)	0.07 (0.04–0.10)
[Hexoestrol]	0.35	0.003]
Dienoestrol monoglucuronide	7.2 (6.5–8.1)	0.15 (0.07–0.3)
[Dienoestrol]	0.75	0.003]

taken up in a small amount of acetone and reprecipitated by addition of ether. The slight amorphous precipitate first formed was removed by filtration, when the monoglucuronide crystallized from the filtrate slowly on standing at 0°. The crystals thus obtained gave a positive Tollens test (after 30 min. acid hydrolysis).

After drying the fine white crystals for 4 hr. at 110° *in vacuo* a white mass of partially fused needles sintering at 85–95° and decomposing at 188–196° was obtained. On drying *in vacuo* (0.1 mm. Hg) at 110° for 4 hr. 7.9 mg. glucuronide lost 0.8 mg. (10.1 %) with some sintering. Theoretical loss for $3H_2O$, 10.8 %. On exposing the dried sample to the atmosphere for 48 hr. at room temperature the glucuronide regained 0.4 mg. in weight. The crystals were soluble in acetone and ethanol, sparingly soluble in ether, insoluble in light petroleum and benzene. $[\alpha]_D^{24} = -73.4^\circ$ ($c = 0.976$ in absolute ethanol). Equivalent weight = 482. Theory for the monohydrate of the monoglucuronide = 464. Found: C, 62.4; H, 6.7. $C_{24}H_{32}O_8$ requires C, 62.2; H, 6.9 %.

Dienoestrol monoglucuronide. This substance also proved difficult to purify since it did not crystallize well and was apparently somewhat unstable. The sodium salt of the glucuronide however crystallized from saturated aqueous bicarbonate solution and was used for purification. The free acid was soluble in acetone and ethanol, sparingly soluble in ether

Biological properties

The oestrogenic activities of the three glucuronides are recorded in Table 1. The activities of the corresponding free oestrogens are given for comparison. Although aqueous glycerol is the preferred solvent for intravaginal tests, arachis oil was used in the reported tests for the sake of uniformity.

DISCUSSION

All the glucuronides have only 5–10 % of the oestrogenic activity of the corresponding free oestrogen. This is true whether the assays are made by subcutaneous or intravaginal methods.

If the activity were due to the complete hydrolysis and liberation of free oestrogen then the glucuronides should show about 50 % of the activity of their parent compounds. In actual fact their activity is about 10 %, calculated on the weight of oestrogen present. The small activity which the glucuronides do show may be due either to their intrinsically low oestrogenic potency, to the liberation of only a proportion of free oestrogen or to more rapid elimination from the organism. That the proportion of free oestrogen liberated, if any, is very small indeed is indicated by the low excretion of free stilboestrol following the administration of its glucuronide (Wilder Smith & Williams, 1948), and is probably not sufficient to account for the biological

activity shown. From evidence so far available it is not, however, possible to formulate the factors to which the lowered potency of the glucuronides is due.

SUMMARY

The isolation and purification of the monoglucuronides of stilboestrol, hexoestrol and dienoestrol,

and their chemical and oestrogenic properties, are described.

We are indebted to the Council of the Middlesex Hospital Medical School for the provision of laboratory facilities for this work, to Dr A. C. Bottomley for help with the statistical side of the bioassays and to Mrs D. Culliford and Mr B. D. Shepherd for technical assistance and to the Medical Research Council for a grant.

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The Preparation and Some Properties of Hyaluronic Acid from Human Umbilical Cord

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Hyaluronic acid has now been known for ten years and in this period the literature about it has become both extensive and inconsistent. A number of substances with varying properties has been isolated by different methods from umbilical cord, skin, vitreous humour, synovial fluid, tumours and haemolytic streptococci. In spite of the differences in their properties, all are called hyaluronic acid if they give more or less viscous solutions and if they are composed for the most part of glucuronic acid and acetylglucosamine. There is no clear evidence whether several substances exist with somewhat similar properties, or whether the observed variations are due to contamination with unrelated material, or to partial destruction of originally homogeneous hyaluronic acid during the isolation.

As a prelude to an investigation of the effect of various agents on the enzymic decomposition of hyaluronic acid we have tried to define the conditions under which a product with more clearly defined properties can be prepared. This was necessary because crude preparations differed greatly in the rates at which they lost viscosity with testis hyaluronidase. Contamination of the preparation by substances activating or inhibiting the enzyme is the most obvious explanation of this phenomenon.

The elementary composition of a hyaluronic acid preparation is of little value as a criterion of purity.

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The likely contaminants have carbon and hydrogen contents similar to those expected for hyaluronic acid. The nitrogen content is a good index of heavy contamination with protein, but no great confidence should be placed on a 'correct' nitrogen value, for this is easily attained by the simultaneous presence of comparable amounts of protein and a nitrogen-free polysaccharide. There is substantial agreement that hyaluronic acid contains neither phosphorus nor sulphur but freedom from sulphur has not often been achieved in making preparations of hyaluronic acid.

Apart from proximate analysis, hyaluronic acid can be examined for its constituents: glucosamine, glucuronic acid and acetic acid; and the equivalent weight can be determined by titration with alkali. These measurements have not often been made on the preparations used as substrate in studies on hyaluronidase. Where they have been made considerable variation is apparent, especially in the case of determinations of the equivalent weight. This value should be 379 for the free acid, on the assumption that the molecule is so large that the ends may be neglected, but Meyer, Smyth & Dawson (1939) and Meyer & Chaffee (1940) have given a series of values ranging from 400 to 640, and our own values are within this range. It seems likely that the carboxyl group in at least part of the glucuronic acid is not free. The existence of this type of linkage robs the equivalent weight of some of its value as a criterion of purity.