Identification of the Uronic Acid from Oestriol 'Monoglucuronide'

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In 1936 Cohen & Marrian isolated from human pregnancy urine an amorphous water-soluble substance containing about 50 % of combined oestriol. From its elementary composition and properties this substance appeared to consist largely of an oestriol monoglucuronide. Subsequently (Cohen, Marrian & Odell, 1936) this substance yielded a crystalline sodium salt, which, on the basis of its elementary composition and a strongly positive Tollens naphthoresorcinol reaction, appeared to be nearly pure sodium oestriol glucuronidate. In the course of this work it was shown 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 hydroxyl group of the latter. No clear-cut evidence was obtained, however, that the uronic acid is indeed glucuronic acid. In view of the lack of specificity of the Tollens reaction for glucuronic acid (Mandel & Neuberg, 1908; Dische, 1946) it was felt that a further investigation of the identity of the uronic acid component should be undertaken.

The identification of uronic acids is difficult, and the choice of method was restricted by the small quantity of purified oestriol 'monoglucuronide' which could be prepared. Unequivocal methods such as the isolation of D-glucurone as used by Pryde & Williams (1933) in the case of borneol glucuronide, or the preparation of the *p*-toluidine complex of ammonium glucuronidate (Smith & Williams, 1949) are not recommended for work with small quantities of glucuronide (Williams, 1949). The method of Lohmar, Dimler, Moore & Link (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 & 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 'monoglucuronide' with reasonable certainty.

The hydrolysis necessary for the liberation of the uronic acid from combination with oestriol presented certain difficulties. The work of Stevenson & Marrian (1947) on the acid hydrolysis of conjugated oestrogens in human pregnancy urine suggested that the complete hydrolysis of oestriol 'monoglucuronide' may require as long as 30–40 min. boiling with addition of 15 % by volume 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 & Marrian (1937) and Fishman (1939) showed that preparations of β-glucuronidase obtained from animal tissues will liberate oestriol from 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.

Sodium oestriol 'monoglucuronidate', purified as thoroughly as existing methods permit, was incubated in two portions with a purified ox-spleen β-glucuronidase preparation, and gave uronic acid fractions in yields of about 62 and 79 % of theory. After oxidation with bromine, and treatment with *o*-phenylenediamine, a product was obtained which was satisfactorily identified as the dibenziminazole of D-glucosaccharic acid. It is clear therefore that the uronic acid obtained by the enzymic hydrolysis of oestriol 'monoglucuronide' 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.

A preliminary communication on this work has already been made (Grant & Marrian, 1948).

EXPERIMENTAL

All melting points referred to in this section are corrected.

Preparation of sodium oestriol 'monoglucuronidate'

Na oestriol 'monoglucuronidate' was prepared from human pregnancy urine by the method of Cohen *et al.* (1936) with various modifications. The troublesome emulsions which

frequently form when urine is shaken with butanol, were avoided by adopting a modification of the procedure of Robinson & Warren (1948). These workers found that emulsions did not form with $CHCl_3$ if it was injected in the form of a fine spray under the surface of the urine which was being extracted. 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 150 cm. long and 4 cm. 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 siphoned 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 urine was collected in batches of 5–10 l., acidified to pH 3·5 with HCl, and extracted with *n*-butanol as described. The pooled butanol extracts were brought to pH 7 with 10% (w/v) NaOH in butanol, concentrated under reduced pressure to $\frac{1}{5}$ volume, and extracted three times with $\frac{1}{10}$ volumes of 0·33 N-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 $\frac{1}{3}$ volumes of butanol. The combined butanol extracts were washed twice with $\frac{1}{10}$ volumes of 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. Crude Na oestriol 'monoglucuronidate' was obtained from the benzene-washed aqueous solution by the quinoline extraction and subsequent procedures described by Cohen *et al.* (1936). The average yield of the crude salt obtained at this stage was 10 mg./l. of urine.

The crude product was purified in the following way: repeated leaching of the solid with hot moist butanol dissolved the Na oestriol 'monoglucuronidate' leaving a small amount of undissolved dark-coloured tar. The combined butanol leachings were extracted three times with $\frac{1}{10}$ volumes of 0·33 N-NaOH; the combined alkaline extracts were acidified to pH 3·5 with HCl, and extracted three times with $\frac{1}{3}$ volumes of butanol. The butanol extract was washed twice with $\frac{1}{10}$ 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 $\frac{1}{5}$ volume. During the concentration Na 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*.

Preparation and characterization of oestriol 'monoglucuronidic' acid

Satisfactory C and H analyses were not obtained with material purified as described above; it was therefore converted to oestriol 'monoglucuronidic' acid as follows. The Na salt was dissolved in a minimum of warm moist butanol. The solution was adjusted to pH 1 with HCl, washed with $\frac{1}{10}$ volumes water until 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 repeatedly wetting with warm water, drying over $CaCl_2$ and subsequently over P_2O_5 *in vacuo* at 80°.

The sample of the acid which was reconverted to the Na salt for enzymic hydrolysis had m.p. 224–226°(decomp.); $[\alpha]_D^{17°} - 7·5° \pm 0·8°$ in ethanol (*c*, 1·061). (Found: C, 62·2; H, 6·9. Calc. for oestriol 'monoglucuronidic' acid $C_{24}H_{32}O_9$: C, 62·0; H, 7·0%.)

Preparation and characterization of sodium oestriol 'monoglucuronidate' for enzymic hydrolysis

The Na salt used for the enzymic hydrolysis was prepared from the pure oestriol 'monoglucuronidic' acid by dissolving the acid in anhydrous methanol, adding an equivalent of NaOH in anhydrous methanol, and evaporating the solution to small volume under an air stream. The white solid which separated was filtered off and washed with cold dry methanol. A sample after drying *in vacuo* at 137° over P_2O_5 for 8 hr. melted at 245–248·5° (decomp.). (Found: C, 59·6; H, 6·5; Na, 4·6. Calc. for Na 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. After drying to constant weight over P_2O_5 *in vacuo* at 137°, a sample picked up 17·1% moisture on equilibration with the laboratory atmosphere.

In the previous work (Cohen *et al.* 1936) preparations of the Na salt containing 0·5 molecule of methanol of crystallization and 1·5 molecules of water of crystallization and melting respectively at 305–306° (uncorr.) and 256–257° (uncorr.) were described, but the solvent-free salt was not obtained. However, in a private communication Dr S. L. Cohen has informed the authors that he also has now prepared the solvent-free Na salt.

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.

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

Na oestriol 'monoglucuronidate' (350 mg.) was incubated at 37° in 250 ml. 0·25 M-acetate buffer, pH 4·6, containing 1000 Graham units of β-glucuronidase. Toluene (1 ml.) was added to inhibit growth of bacteria. A further 500 units of enzyme in 45 ml. solution were added after 24 hr. At the end of 3 days protein was precipitated with 4 vols. of dry acetone, and filtered off. The filtrate was evaporated to a volume of 100 ml. It was then acidified with H_2SO_4 and extracted 3 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 'glucuronide'. This uronic acid fraction was oxidized, and the dibenziminazole derivative of the product was prepared 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. Na 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 the 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 $[\alpha]_D^{18°} + 49{\cdot}8° \pm 0{\cdot}6°$ in water (*c*, 2·057). On admixture with an authentic sample (m.p. 266–267°; $[\alpha]_D^{18°} + 49{\cdot}3° \pm 0{\cdot}6°$ in water (*c*, 2·042)) the m.p. was unchanged. Levvy (1948) found $[\alpha]_D^{18°} + 52{\cdot}3°$ 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 the dihydrochloride isolated from the urinary uronic acid and the authentic dihydrochloride were recrystallized from water, and dried over $CaCl_2$, under identical conditions.

SUMMARY

1. A modified method for the isolation and purification of oestriol monoglucuronidic acid and its sodium salt from human pregnancy urine is described.

2. The sodium salt has been hydrolysed by ox-spleen β-glucuronidase. 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.

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Studies on Rhodopsin

1. METHODS OF EXTRACTION AND THE ABSORPTION SPECTRUM

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The history of visual pigments has been reviewed on numerous occasions: Granit (1947) has given a comprehensive and invaluable summary. As the result of the work of R. J. Lythgoe and his co-workers and of G. Wald, our knowledge of rhodopsin has been greatly enlarged. This knowledge can be conveniently summarized in the cycle shown on p. 4, based on that first proposed by Wald (1938).

It has been shown that rhodopsin is responsible for scotopic or twilight vision although not for photopic vision. Retinene has been shown in this laboratory (Morton & Goodwin, 1944) to be vitamin A aldehyde, whilst indicator yellow is now thought to be a compound between retinene and a suitably bound amino group (Ball, Collins, Dalvi & Morton, 1949) of the protein moiety.