The Isolation of Pregnane-3 α :17 α :20 α -triol from the Urine of Normal Men

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There are reasons for supposing that C₂₁ 17:20-dihydroxy-20-methyl steroids may be among the urinary metabolic reduction products of the C₂₁ 17-hydroxy adrenocortical hormones (cf. Marrian, 1951*a, b*; Cox, 1952). Two steroids of this type have been isolated from the urine of human subjects with adrenal hyperplasia or tumour—pregnane-3 α :17 α :20 α -triol (Butler & Marrian, 1937, 1938; Mason & Kepler, 1945; Miller & Dorfman, 1950), and pregn-5-ene-3 β :17 α :20 α -triol (Hirschmann & Hirschmann, 1950), but hitherto none has been isolated from the urine of normal human subjects.

Recently Cox (1952) has developed a method, using oxidation with periodic acid, for the quantitative determination in urine extracts of 'acetaldehydogenic' C₂₁ 17:20-dihydroxy-20-methyl steroids, and with its aid has detected the presence of acetaldehydogenic material in alkali-washed chloroform extracts of unhydrolysed urine of normal men. After hydrolysis of the urine at pH 1.0 and 25° for 24 hr. the amounts of extractable acetaldehydogenic material were significantly increased, while hydrolysis with crude ox-spleen β -glucuronidase caused a greater increase up to values equivalent to about 1–2 mg. of steroid per 24 hr. In view of the possibility that this acetaldehydogenic material might be steroidal in nature it was decided to investigate its chemical nature.

RESULTS

From pooled urine from healthy young men a concentrate was prepared by chloroform extraction following hydrolysis with ox-spleen β -glucuronidase. The acetaldehydogenic material in this concentrate was further concentrated by partition between aqueous methanol and benzene on a Super-cel column. From this chromatographically purified fraction a crystalline substance, m.p. 247–248°, was isolated, which on oxidation with periodic acid gave acetaldehyde in almost theoretical yield for that required for the oxidation of a 17:20-dihydroxy-20-methyl steroid of the formula C₂₁H₃₆O₃.

The identity of this substance with pregnane-3 α :17 α :20 α -triol was established by mixed melting points of the substance and its acetate with authentic specimens of the triol and its acetate respectively.

In a previous paper (Cox & Marrian, 1953) it was pointed out that the liberation of unconjugated adrenocortical steroids on incubation of urine with crude preparations containing β -glucuronidase does not provide satisfactory evidence that these substances arise by the hydrolysis of urinary glucuronides by β -glucuronidase itself. It was shown, however, that the liberation of both formaldehydogenic and acetaldehydogenic steroids on incubation of butanol-extractable material from urine with ox-spleen β -glucuronidase preparations is largely inhibited if the incubation is carried out in the presence of saccharate (cf. Karunairatnam & Levy, 1949). Accordingly, it was concluded that the greater part of the formaldehydogenic and acetaldehydogenic steroids in urine are, in all probability, present as their glucuronides. In the present work, however, the pregnane-3 α :17 α :20 α -triol actually isolated only accounted for a small fraction of the total acetaldehydogenic material present in the urinary concentrate, and accordingly it could not be assumed that the triol had been liberated by the enzymic hydrolysis of its glucuronide, nor could it be assumed that it was the only acetaldehydogenic substance present in the urine concentrate.

To investigate these points a further experiment was carried out in which the butanol-soluble fraction of men's urine was incubated with ox-spleen β -glucuronidase preparations. Partition between aqueous methanol and benzene on a Super-cel column of a portion of the alkali-washed chloroform-soluble material from the incubation mixture revealed the presence of three quite distinct groups of acetaldehydogenic substances. The elution pattern of the one present in the largest amount was very similar to that of pregnane-3 α :17 α :20 α -triol, and the latter was subsequently isolated from the chromatographically separated pregnanetriol-like fraction from the main bulk of the extract. There is little doubt, therefore, that this acetaldehydogenic fraction consists largely, if not entirely, of pregnane-3 α :17 α :20 α -triol, and the chromatographic data suggest that in this particular extract from men's urine it was present in amounts equivalent to about 1.3 mg./24 hr. The other two chromatographically-separated acetaldehydogenic fractions have not yet been further investigated.

In this experiment the incubations with ox-spleen β -glucuronidase were strictly controlled by parallel incubations using boiled enzyme, and unboiled enzyme in the presence of saccharate. Extracts from these two types of control incubations were also partitioned using the same solvent system, and in this way it was shown that the liberation of all three groups of acetaldehydogenic substances was markedly depressed in both instances. This finding provides strong evidence to indicate that all these groups of acetaldehydogenic substances are present in urine largely as glucuronides. The inhibition by saccharate was, however, apparently much greater in the case of the pregnanetriol fraction than it was for the other two; and thus the possibility must be considered that the latter may be present in urine, not only as glucuronides, but also as conjugates of some other type.

EXPERIMENTAL

General. Ox-spleen β -glucuronidase was prepared and standardized by the methods previously described (Cox, 1952).

Except where otherwise stated, all evaporations were carried out under reduced pressure.

Melting points were determined on an improvised microscope hot stage (Klyne & Rankeillor, 1947), which was previously standardized with pure compounds of known melting point, and are corrected for emergent stem.

The procedure for preparing columns for partition chromatography was that of Butt, Morris, Morris & Williams (1951) with the following two exceptions: (i) In the purification of Hyflo Super-cel (Johns-Manville) the suspension in HCl was not boiled. (ii) In packing the columns the Super-cel was mixed with the stationary phase in the proportion of 1 g./1 ml. instead of 1.5 g./1 ml. All operations involved in the chromatography, including equilibration of solvents and packing of columns, were carried out in a constant temperature room at $17.5^\circ \pm 0.5^\circ$. Preliminary experiments with authentic pregnane-3 α :17 α :20 α -triol indicated that the solvent systems 70% (v/v) aqueous methanol-benzene and 80% (v/v) aqueous methanol-benzene might be suitable for the fractionation of the urinary acetaldehydogenic substances.

First experiment

Hydrolysis and extraction of urine. Urine was collected from male medical students, using CHCl_3 as preservative, acidified to pH 2.0 with 10N- H_2SO_4 and extracted once with 2 vol. of CHCl_3 in batches of 4–10 l. to remove unconjugated and 'CHCl₃-soluble conjugated' steroids (cf. Paterson, Cox & Marrian, 1950; Paterson & Marrian, 1951). The extracted urine was neutralized to pH 5.0 with Na_2CO_3 solution and incubated at 37° for 20–24 hr. after the addition of β -glucuronidase (10 000 units/l.). (When this experiment was carried out it was believed that the optimum pH for the hydrolysis of the conjugated urinary adrenocortical steroids by ox-spleen β -glucuronidase was about 5.0. Later work has shown, however, that higher yields of both formaldehydogenic and acetaldehydogenic steroids are obtained when the enzymic hydrolysis is carried out at pH 4.5.) The incubation

mixture was extracted with CHCl_3 (once with 0.33 vol. and twice with 0.75 vol.), and the combined extract washed three times with 0.1 vol. N-NaOH and three times with 0.1 vol. water. After drying over anhydrous Na_2SO_4 the washed extract was evaporated to dryness, the residue dissolved in benzene (20 ml./l. urine extracted), and the benzene solution stored at 0° .

When 68 l. of urine had been worked up in this way the cold benzene solutions were filtered from small amounts of insoluble material, and the combined filtrates evaporated to dryness. The resulting reddish gum, weighing 3.07 g., was dissolved in 100 ml. aqueous 70% (v/v) ethanol and the solution extracted four times with 0.6 vol. *n*-hexane. The hexane extract was discarded, and the aqueous ethanolic phase evaporated to about 20 ml. and extracted four times with 2 vol. CHCl_3 . Bad emulsions were formed in this extraction and accordingly the aqueous phase was re-extracted three times with 4 vol. CHCl_3 after dilution with water to 100 ml. The combined CHCl_3 extracts were dried over Na_2SO_4 and evaporated to dryness. The product was a reddish oil weighing 1.67 g.

Isolation and identification of pregnane-3 α :17 α :20 α -triol. Slightly more than half of the above-mentioned concentrate was used up in abortive attempts at the direct isolation of crystalline material. The remainder, weighing 0.75 g., was found to contain acetaldehydogenic material in an amount equivalent to about 50 mg. of pregnane-3 α :17 α :20 α -triol.

This material was partitioned on a Super-cel column (35.5 cm. $\times$ 3.14 sq. cm.) using the solvent system 70% (v/v) aqueous methanol-benzene and a rate of flow of 15 ml./hr. It was transferred to the column in 5 ml. of the mobile phase, and eluate fractions (in most cases 10 ml.) were collected, evaporated under an air stream at 60° , dried in a desiccator over CaCl_2 , and weighed. Each residue was dissolved in 5.0 ml. ethanol, and 0.050 ml. samples of each solution withdrawn for the determination of acetaldehydogenic substances. The elution curves, shown in Fig. 1, show that a considerable degree of concentration of acetaldehydogenic material in certain of the fractions was achieved.

The fractions eluted by 95–267 ml. of the mobile phase were combined and repartitioned on a similar column using the same solvent system. No appreciable further concentration of acetaldehydogenic material was achieved, and accordingly attempts were made to isolate crystalline material directly.

The first 117 ml. of eluate from this second chromatogram were rejected, and then volumes of 40, 40, 60 and 50 ml. were separately evaporated under an air stream and treated with methanol-benzene. Crystalline products melting over the range 241 – 249° were obtained from each of the first two fractions, and these when combined and recrystallized from 80% (v/v) aqueous methanol yielded about 3 mg. of white needles, m.p. 248 – 249° . The mixed m.p. with authentic pregnane-3 α :17 α :20 α -triol (m.p. 247 – 248°) was 247 – 248° .

The second two fractions from the second chromatogram, which yielded no crystals on treatment with methanol-benzene, were combined with the mother liquors from the above-mentioned crystallizations, and this material in CHCl_3 solution was poured on to a magnesium silicate-Celite column (1:1). The chromatogram was developed with CHCl_3 and then eluted successively with 2 and 4% (v/v) ethanol in CHCl_3 . Those fractions showing the presence of crystalline material on moistening with benzene were combined and washed with a small volume of cold acetone. The whole solid remaining was crystallized, once from

aqueous ethanol and twice from 80% (v/v) aqueous methanol, yielding a further 2.4 mg. of white needles, m.p. 246–247°. (Found: C, 74.2; H, 10.7. Calc. for C₂₁H₃₆O₃: C, 75.0; H, 10.8%.)

Oxidations in triplicate were carried out with 25.3 μ g. amounts of the crystalline substance (m.p. 248–249°), and 3.36, 3.53 and 3.35 μ g. acetaldehyde were obtained. The calculated amount of acetaldehyde which would be yielded by 25.3 μ g. of pregnane-3 α :17 α :20 α -triol is 3.31 μ g.

About 2 mg. of the substance (m.p. 248–249°) were acetylated by heating at 100° for 2 hr. with 0.5 ml. acetic anhydride and 0.05 ml. dry pyridine. After removal of the excess reagents by treatment with ice-cold water, the crude product was crystallized once from 50% (v/v) aqueous ethanol and once from light petroleum (boiling range

preservative, and separately extracted with butanol. The butanol-soluble fraction from each was divided into three equal portions, A, B and D. These were incubated in aqueous solution at pH 4.5 (acetate buffer) for 48 hr. at 37°, A with 5000 units of ox-spleen β -glucuronidase, B with 5000 units of β -glucuronidase which had been boiled, and D with 5000 units of β -glucuronidase in the presence of 8×10^{-3} M-saccharate. The incubation mixtures were then extracted with CHCl₃, and the extracts from each washed with NaOH solution and with water, dried over Na₂SO₄ and evaporated to dryness. (For details see Cox & Marrian, 1953.)

Partition chromatography of the extracts. The twelve extracts from the A portions were combined, as were also those from the B and D portions. Nine-tenths of each of the

Fig. 1. Partition chromatogram on 35.5 cm. $\times$ 3.1 sq. cm. Super-cel column of a urine extract obtained after incubation of urine with crude ox-spleen β -glucuronidase. Solvent system: 70% (v/v) aqueous methanol-benzene.

40–60°), yielding crystals melting at 148–149° and 158–159°. Mixed with authentic pregnane-3 α :17 α :20 α -triol 3:20-diacetate (m.p. 147–149° and 156–159°) the m.p.'s were 147–148° and 155–157°.*

The identification of the isolated substance as pregnane-3 α :17 α :20 α -triol was considered to be complete.

Second experiment

Hydrolysis and extraction of urine. Twelve 24 hr. urine specimens were collected from normal men using butanol as

* The melting behaviour of pregnane-3 α :17 α :20 α -triol 3:20-diacetate is peculiar and is reminiscent of that of pregnane-3 α :20 α -diol diacetate (cf. Verly, Sommerville & Marrian, 1950). The lower melting point is not invariably seen; sometimes the substance undergoes only a change of crystalline form at this temperature. By reheating melted and supercooled samples, which had been induced to solidify at various temperatures by scratching, evidence was obtained to indicate that pregnanetriol diacetate can exist in two other polymorphic modifications which melt or undergo change of form at about 73–78° and 136°. These observations would seem to explain the discrepancies in the literature concerning the melting point of this substance (cf. Butler & Marrian, 1937, 1938; Mason & Kepler, 1945; Klyne, 1949.)

three combined fractions were set aside for subsequent isolation work, while the remaining one-tenth portions were used for determinations of total acetaldehydogenic substances and for quantitative partition chromatograms.

Portions of each of the combined extracts (1.2% of A, 6% of B and D) were partitioned on a Super-cel column (10 cm. $\times$ 1.0 sq. cm.) using the solvent system 80% (v/v) aqueous methanol-benzene and a flow rate of 7–10 ml./hr. Eluate fractions of 2.0 ml. were collected, evaporated under an air stream at 60°, and assayed for acetaldehydogenic substances. Material not eluted from the column by the mobile phase was washed out with methanol and also assayed for acetaldehydogenic substances.

The results of this experiment are shown graphically in Fig. 2. It will be seen that the acetaldehydogenic material in the A extract was eluted from the column by the mobile phase in two distinct bands, the more 'polar' of which behaved chromatographically like pregnane-3 α :17 α :20 α -triol (cf. Fig. 3). Further acetaldehydogenic material was washed out of the column by methanol, and presumably this represents a third and still more polar type of compound. It will also be seen that the liberation of all three types of acetaldehydogenic substances during incubation was markedly inhibited by boiling the enzyme preparation or by the presence of saccharate.

Isolation and identification of pregnane-3 α :17 α :20 α -triol. The remaining nine-tenths of the A concentrate was

partitioned on a Super-cel column (20 cm. $\times$ 3.14 sq.cm.) between 80% (v/v) aqueous methanol and benzene using a flow rate of 30 ml./hr. From the ratio of the dead volumes of this column and the one used for the partitions described in the preceding section it was calculated that the greater part of the pregnanetriol-like material would be present in the fractions eluted by 95–176 ml. of mobile phase. The eluate between these volumes was evaporated to dryness, and the residue washed with a small volume of cold acetone.

Fig. 2. Partition chromatograms on 10 cm. $\times$ 1.0 sq.cm. Super-cel columns of urine extracts after incubation with (A) β -glucuronidase, (B) boiled β -glucuronidase, (D) β -glucuronidase in presence of 8×10^{-3} M-saccharate. Solvent system: 80% (v/v) aqueous methanol-benzene. After eluate volumes indicated $\downarrow$, substances remaining on the columns were washed off with methanol ('methanol wash').

The white solid obtained, after crystallizing twice from aqueous methanol and washing with a few drops of cold acetone, yielded about 1.5 mg. of needles, m.p. 240–245°. About 0.5 mg. of this was crystallized twice from acetone-hexane, yielding crystals melting at 245–248°. Mixed with authentic pregnane-3 α :17 α :20 α -triol the m.p. was 245–246°.

The remainder of the crude crystalline product was acetylated in the manner previously described. After crystallization from 50% (v/v) aqueous methanol and from light petroleum (boiling range, 40–60°) the product yielded crystals melting at 143–146° and 156–157°. Mixed with

authentic pregnane-3 α :17 α :20 α -triol 3:20-diacetate the m.p.'s were 145–147° and 156–159°.

The identification of the isolated substance as pregnane-3 α :17 α :20 α -triol was considered to be complete.

Fig. 3. Partition chromatogram on 10 cm. $\times$ 1.0 sq.cm. Super-cel column of pure pregnane-3 α :17 α :20 α -triol. Solvent system: 80% (v/v) aqueous methanol-benzene.

SUMMARY

1. Pregnane-3 α :17 α :20 α -triol has been isolated from the urine of normal men after hydrolysis with ox-spleen β -glucuronidase.

2. Partition-chromatographic evidence has been obtained to indicate the presence in the urine of normal men of two types of acetaldehydogenic substances besides pregnane-3 α :17 α :20 α -triol.

3. These three acetaldehydogenic substances are probably present in urine largely in the form of their glucuronides, since their liberation on incubation with ox-spleen β -glucuronidase preparations is markedly inhibited in the presence of saccharate.

4. The chromatographic evidence suggests that the urine of normal men may contain about 1–2 mg. pregnane-3 α :17 α :20 α -triol/24 hr.

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