Conjugation of Androsterone by the Guinea-Pig Kidney *in vitro*

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Radioactive androsterone was incubated with kidney slices from guinea pigs in a Ringer bicarbonate buffer. For analysis, the radioactivity was partitioned between chloroform and water for the separation of non-polar and polar steroids. After multiple chromatography procedures, the non-polar fraction was shown to contain androsterone and at least four other similar steroids, not identified in this study. The polar fraction yielded numerous conjugates from which androsterone glucuronide and androsterone sulphate in approximately equal quantities were isolated and characterized.

It has been shown that dog kidney is capable of conjugating androsterone and aetiocholanolone *in vivo* (Cohn & Hume, 1960), and that guinea-pig kidney is very active in conjugating certain functional groups *in vitro* (Stevenson & Dutton, 1962). The present study was undertaken to explore the ability of the kidney to conjugate C₁₉ steroids *in vitro*.

MATERIALS AND METHODS

General procedure. Erlenmeyer flasks (50 ml. capacity) were prepared for incubation by adding 2 μ C of [³H]-androsterone dissolved in ethanol and drying the flasks under a flow of air. [³H]Androsterone prepared by the Wilzbach (1957) procedure was purified by solvent partitioning, by paper and column chromatography and by derivative formation, and constant specific radioactivity was observed after the reverse isotope dilution. The specific radioactivity was 2.6 μ C/ μ g. (Wilzbach, 1957). After complete drying of the flasks, 20 ml. of Ringer bicarbonate buffer (Umbreit, Burris & Stauffer, 1964), pH 7.4, was added to each flask and vigorously agitated to dissolve the radioactive androsterone (the quantity used had been previously demonstrated to be soluble in 20 ml. of buffer solution). The flasks were then brought to 37° in a Dubnoff incubator for receipt of the tissue. Adult male guinea pigs were killed by a sharp blow to the base of the skull. Immediately the diaphragm, chest wall and abdominal wall were sectioned wide enough to expose both kidneys in rapid sequence. The kidneys were removed by blunt dissection and slices made with the Stadie-Riggs microtome while they were chilled. The slices were kept chilled until randomization and then, after being weighed (approx. 300 mg./flask), they were placed in the incubation mixture under an atmosphere of O₂ + CO₂ (95:5) for aerobic incubation and N₂ + CO₂ (95:5) for anaerobic incubation. Additional sections were weighed and placed for 1 min. in boiling water; there was obvious coagulation of the protein.

The incubations were terminated at 2 hr. by the addition of chloroform, and extraction was carried out by using volumes of chloroform twice those of the incubation

mixtures. Extraction was continued until a drop was free of radioactivity. All samples were treated in an identical manner. All samples were extracted 40 times. The radioactivity of chloroform component was counted and subjected to paper chromatography, with as a control non-radioactive androsterone, obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). The water component was likewise counted and subjected to countercurrent distribution, paper electrophoresis, column and paper chromatography and selective hydrolysis, with as a control non-radioactive androsterone glucuronide, obtained through the courtesy of Dr John Schneider, and androsterone sulphate, obtained from Southern Biochemical (Morristown, Tenn., U.S.A.).

Measurement of radioactivity. Radioactivity was measured in a Packard Tri-Carb model 314-EX scintillation counter with a ³H-counting efficiency of 18%. Localization was done with a Nuclear-Chicago strip scanner. All determinations were carried out within the 3% confidence level.

In view of the type of tissue and its subsequent exposure to a variety of non-sterile conditions, the procedure was monitored with bacterial cultures, but no contamination was found.

Paper chromatography of the free compounds. The descending system with heptane-methanol-water (20:19:1, by vol.) was used. The equilibrated two phases were added in appropriate fashion to the paper-lined chromatography tank (75 cm. $\times$ 30 cm.) with stainless-steel supporting racks, which was closed and given 12 hr. for equilibration. Samples and the unlabelled control androsterone were transferred with chloroform to the Whatman no. 1 chromatography paper, which was then placed in the tank. After equilibration for 4 hr., the mobile phase was added and allowed to migrate for 12 hr. The paper was removed, air-dried and carried through location analysis for radioactivity and chemical compounds.

Countercurrent distribution. Each column of the apparatus, a 20-tube hand model made by E-C Apparatus Corp. (Philadelphia, Pa., U.S.A.), was filled with 10 ml. of both phases of aq. 0.02% ammonia-ethyl acetate-butanol (2:1:1, by vol.). The sample was completely transferred with 10 ml. of each phase to the first column. After 10 min.

of slow agitation, the apparatus was placed in the rest position, allowing the establishment of a sharp interface. After rotation and transfer of the upper phases, mixing was begun. Nineteen transfers were carried out. The apparatus was emptied into test tubes corresponding to each column. These tubes were evaporated under air to dryness and the contents were resuspended in 10 ml. of methanol. Samples were taken and dried in counting vials, scintillation fluid was added and the radioactivity was determined with the scintillation counter.

Electrophoresis. Paper strips, 3-8 cm. wide, were saturated in 0.02M-sodium veronal buffer, pH 8.4, and blotted. After the strips had been mounted in an electrophoresis tank (model E-800-2; Apparatus Research Corp., Philadelphia, Pa., U.S.A.), the closed system was allowed to equilibrate at 24° for 4 hr. Each sample was streaked at the cathode origin, along with unlabelled controls (androsterone glucuronide and androsterone sulphate) on an adjacent strip. After a total time of 18 hr. had been allowed for migration with 8 Δ and 250 v applied across a total of 7.5 cm. of paper, the strips were removed and dried while suspended horizontally. The radioactivity was located by running the strips through the strip scanner with 1.3 cm. slit and maximum sensitivity settings. The radioactive areas were compared with the unlabelled controls, which had been located by spraying with Zimmermann reagent (Smith, 1960). Before each run, the pH of the buffer was established at 8.4, and the same number of strips was always used.

Column chromatography of conjugates. Florisil, activated at 100° for 4 hr., was added to a column (6 mm. $\times$ 250 mm.) filled with benzene, and allowed to settle by gravity. The samples were transferred to the column with 2 drops of ethanol and elution was begun with consecutive additions of 5 ml. portions of the following: (1) benzene, four lots; (2) benzene-ethyl acetate (1:1, v/v), two lots; (3) ethyl acetate, two lots; (4) ethyl acetate-ethanol (9:1, v/v), five lots; (5) ethyl acetate-ethanol (1:9, v/v), five lots; (6) ethanol, four lots; (7) ethanol-water (9:1, v/v), two lots; (8) ethanol-water (1:1, v/v), two lots; (9) ethanol-water (1:9, v/v), two lots; (10) water, four lots. The sulphate appeared in the fourth and fifth solutions (fraction a) and glucuronide in the seventh, eighth and ninth solutions (fraction b). All free steroids were eluted with the initial additions of benzene.

Paper chromatography of conjugates. The descending system with ethyl acetate-hexane-acetic acid-water (6:4:3:7, by vol.) was used. Solvent and tank preparations were identical with those for free compounds.

The dried conjugated samples were suspended in ethanol and spotted on to paper (15 cm. $\times$ 55 cm.), which was then cut into 2.5 cm. 'fingers'. Unlabelled controls (androsterone glucuronide and androsterone sulphate) were spotted in similar fashion on adjacent 'fingers'. The paper was placed into the tank and, after 4 hr. of equilibration, mobile phase was added and allowed 8 hr. for migration. After removal of the paper from the tank and drying it in air, the radioactivity was located by scanning and the compounds were located by staining.

Selective hydrolysis. β -Glucuronidase containing no sulphatase activity (obtained as Ketodase from Warner-Chilcott, Laboratories, Morris Plains, N.J., U.S.A.) was added (0.01 ml.) with 1.0 ml. of sodium acetate buffer, pH 5.0, to each sample of conjugated compound, previously dried in a 12 ml. centrifuge tube. The combined incubation

mixture was agitated in a Dubnoff shaker for 3 hr. at 37°. After the incubation a double volume of chloroform (approx. 2 ml.) was added to each tube. By bubbling air through a capillary tube into the bottom chloroform layer it was mixed with the upper layer. After 1 min. of mixing, separation was allowed until a sharp interface was present. The bottom layer was removed and transferred to 12 ml. graduated centrifuge tubes with a capillary pipette, the procedure being repeated three times. The chloroform portion then was made up to exactly 10 ml. and a portion taken from each tube and dried in a counting vial. Scintillation fluid was added and the samples were counted in the scintillation counter. If there was chloroform-extractable material the radioactivity was compared with the radioactivity in the original conjugated sample, thus allowing determination of the completeness of hydrolysis. The samples that had chloroform-extractable radioactivity were then chromatographed with unlabelled standards and subsequently with the original chloroform-extractable portions. Those samples that had no radioactivity in the chloroform-extractable portions were discarded and their water-soluble counterparts were dried in air and analysed by the paper-chromatography system, with as controls a portion of the original sample that had not been subjected to β -glucuronidase, and another portion that had been subjected only to the acetate buffer, pH 5.0. This was done because sulphates tend to hydrolyse at acid pH. Also, unlabelled controls (androsterone glucuronide and androsterone sulphate) were treated in a similar fashion.

Solvolysis. The procedure outlined by Schneider & Lewbart (1956) was used. The chromatography tank was lined with filter paper and saturated with dioxan-water (1:9, v/v) and allowed to equilibrate for 4 hr. The dried samples of conjugates were suspended in ethanol and spotted on chromatography paper prepared in the usual way. The paper was then placed in the equilibrated tank and left for 3 hr. The combined eluates were evaporated to dryness, the residue was partitioned between chloroform and water, and the chloroform phase was examined as with the β -glucuronidase hydrolysis.

RESULTS

Solvent extraction (Table 1). The total recovery of radioactivity obtained by adding that contained in the water-soluble fraction to that contained in the chloroform-soluble fraction from a partition experiment was complete ($99 \pm 2\%$ of the amount originally added). The partitioning between chloroform and water, however, raises questions. Although the amount of radioactivity in the water component never exceeded 10% of the total radioactivity added, there were variations within the three groups. The aerobic group, when pooled, had the largest water component, but individual samples of the aerobic and anaerobic did overlap. None of the individual boiled samples extended into their range. A variation between the radioactive samples was not unexpected, since the total weight of kidney slices in each flask was only approximately known, and also, although attempts were always made to randomize the material sectioned,

no attempt was made to separate the renal cortex from the medulla. The fact that there was a measurable water component with boiled slices was perplexing until it was demonstrated by chromatography to be unconjugated steroid. This probably represented steroid carried into the aqueous phase in chloroform, which is soluble in water to the extent of 1%. Some of the unconjugated starting material may also have been bound to the protein of the kidney slices retained in the water component and not eluted by the chloroform. To assume, then, that the quantity of radioactivity in the water component of the aerobic and anaerobic samples represented solely conjugated material would be an error. However, only trace amounts of free material were found in the radioactive sample groups, so this problem was not important quantitatively.

Paper chromatography of the chloroform-soluble steroids (Fig. 1). The chloroform-extractable component of the original incubation mixture contained a number of non-polar compounds. Androsterone was located by using unlabelled

carrier as well as a control on adjacent 'fingers'. There remained at least four unidentified compounds as well as the questionable area in the vicinity of the origin. Although the compounds were unidentified, their mobilities suggested that they possessed certain radicals that could be conjugated; hence special precautions were required in interpretation of the water-soluble component. At no time could conjugates be identified in the non-polar component.

Countercurrent distribution of the water-soluble fraction (Fig. 2). The radioactivity was distributed into two peaks, A and B, corresponding to the unlabelled controls, androsterone glucuronide and androsterone sulphate, respectively. On selective hydrolysis radioactive androsterone was recovered

Table 1. Chloroform-water partition of radioactivity from the mixture after incubation of kidney slices with [³H]androsterone

After incubation of slices with 2μC of [³H]androsterone, the entire sample was partitioned between chloroform and water. Each of the three groups was composed of three samples; means are given; the individual results are shown in parentheses; for details of the treatments, see the Materials and Methods section.

Treatment	10 ⁻³ × Radioactivity (c.p.m.)	
	Chloroform range	Water range
Boiled	790 (781, 788, 802)	10 (8, 10, 11)
Anaerobic	768 (760, 767, 777)	40 (30, 39, 45)
Aerobic	752 (747, 753, 756)	49 (44, 51, 53)

Fig. 2. Twenty-tube countercurrent distribution of the radioactivity in the water-soluble component in aq. 0.2% ammonia-ethyl acetate-butan-1-ol (2:1:1, by vol.) forms two peaks, A and B. The distribution of the simple glucuronide and sulphate (Schneider & Lewbart, 1959) is demonstrated by the bars at columns 6-14 and 16-19 respectively. ●, Anaerobic; □, aerobic; x, boiled tissue.

Fig. 1. Radioactivity in the chloroform-soluble component after paper chromatography in heptane-methanol-water (20:19:1, by vol.): trace from Nuclear-Chicago strip scanner. The area d corresponds to the unlabelled androsterone control; the remaining areas are unidentified.

Fig. 3. After countercurrent distribution of radioactivity from the water component into two areas, *A* and *B* (Fig. 2), paper electrophoresis of each was carried out. The upper and lower tracings from the Nuclear-Chicago strip scanner represent areas *A* and *B* respectively.

from each area. However, about 50% of the radioactivity was resistant to hydrolysis under conditions that completely hydrolysed the unlabelled controls.

Paper electrophoresis (Fig. 3). The radioactive substances corresponding to areas *A* and *B*, obtained from countercurrent distribution, were subjected to electrophoresis, and each yielded an area, *A*₁ and *B*₂, that corresponded to the chemical controls, androsterone glucuronide and androsterone sulphate respectively, and on selective hydrolysis each yielded radioactive androsterone.

Column chromatography of the aqueous component. Approx. 1000 c.p.m. of the water component after the initial chloroform partition was subjected to stepwise elution from a Florisil column. Fractions (a) and (b) (see the Materials and Methods section) corresponded to androsterone sulphate and androsterone glucuronide controls, and yielded 290 and 390 c.p.m. respectively. On hydrolysis they yielded the parent androsterone moiety. A benzene component was demonstrated to be a free contaminant of the original partition. There was residual radioactivity on the column.

Paper chromatography of the aqueous phase (Fig. 4). After the countercurrent distribution of radio-

activity into two areas, *A* and *B*, each of these was separately chromatographed and yielded further subdivisions. They are collectively numbered in order of increasing mobility. Areas 2 and 4 corresponded to the unlabelled controls, androsterone glucuronide and androsterone sulphate, and on selective hydrolysis yielded radioactive androsterone.

Selective hydrolysis (Table 2). From preliminary trials it became apparent that the samples obtained by elution after paper chromatography would provide the best general results for demonstrating that the kidney slices had indeed formed two classes of conjugates. Therefore the five radioactive areas were eluted and numbered in sequence from the origin. Amounts having approximately equal radioactivities were subjected to the selective hydrolysis. Areas 1 and 2 were completely resistant to glucuronidase whereas areas 3 and 4 were seen after solvolysis; however, areas 3 and 4 showed some evidence of hydrolysis after the solvolytic process, but the control androsterone glucuronide was likewise hydrolysed to the same extent. Peak 5 was completely extractable before hydrolysis and was therefore considered to be a non-conjugated

Fig. 4. After countercurrent distribution of radioactivity from the water component into two areas, A and B (Fig. 2), paper chromatography of each yielded the upper and lower tracings on the Nuclear-Chicago strip scanners respectively. The areas are numbered, collectively, in order of increasing mobility.

Table 2. *Chloroform component after selective hydrolysis of water-soluble compounds*

After paper chromatography of the aqueous phase to locate simple glucuronides, each of the radioactive areas, numbered 1-5 in order of increasing mobility, was eluted and amounts yielding approx. 800 c.p.m. were subjected to selective hydrolysis. Radioactivities recorded are those found in the chloroform phase of the chloroform/water partition, which indicates that the conjugates had been broken down to yield their corresponding steroids. Areas 2 and 4 corresponded to the androsterone conjugate controls and on hydrolysis yielded the parent androsterone.

Area ...	Radioactivity in chloroform phase (c.p.m.)				
	1	2	3	4	5
Compound (alone)	0	0	0	0	734
Compound and buffer	0	0	0	0	—
Compound and β -glucuronidase	0	0	500	515	—
Compound and solvolysis	806	760	170	210	—

contaminant. This correlates with the observations during other procedures (see 'Column chromatography of conjugates'). This free component was also found at the solvent front and in this respect

behaved as a non-polar steroid. To exclude the presence of diconjugate or mixed-conjugate formation, all the radioactive residues were chromatographed with the original material isolated from the water component and chloroform component. In all cases when there was a water-extractable radioactive residue this material remained unchanged, and when there was chloroform-soluble material after hydrolysis it was found also in the original chloroform-extractable portion. This was taken as evidence for simple conjugate formation and tended to exclude the presence of diconjugates and mixed conjugates.

After all hydrolyses, chemical carrier androsterone was added to the suspected radioactive androsterone, which was subsequently characterized by paper and column chromatography.

The substance obtained as peak 2 from the paper-chromatography system, which yielded androsterone sulphate, was found after solvolysis to give a mixture of androsterone and one of its metabolites, which was not identified. Since the proportions within the individual samples varied in a random fashion one could only say that this peak represented two conjugates and approximately half gave androsterone after hydrolysis. This phenomenon

was also found in group A with substances that formed peaks in the glucuronide area. In this instance, however, androsterone glucuronide was by far the major component and its contaminant was material from an overlapping peak.

DISCUSSION

This investigation was originally designed to demonstrate the ability of the guinea-pig kidney to conjugate the steroid androsterone. This seemed feasible, as androsterone is considered to be a terminal catabolite of androgen metabolism. The analysis would thus show two groups of compounds: the recovered starting material in a chloroform component, and the conjugates, if formed, in a water component after chloroform and the incubation mixture had been shaken together. It would then be possible to account for the radioactivity, and to evaluate the processes quantitatively. The discovery of the ability of the guinea-pig kidney to convert androsterone into a variety of components, also capable of being conjugated, gave tremendous complexity to the analysis, set certain limitations on the quantitative interpretations and required procedures to permit reasonable certainty about the qualitative aspects.

Chloroform extraction yielded a variety of free steroids along with the original androsterone. These represented true metabolic alterations of the androsterone substrate, since the original material was demonstrated to be pure by a variety of procedures, and the boiled controls yielded only the original compound, androsterone. Although there was some variation between the various samples in the aerobic group, one could estimate that the products represented about 80% of the original androsterone. They were involved also in the conjugation procedure and contributed to both components of the original partition. Formal proof of structure of these free steroids was impossible, since the entire experiment was done by tracer analysis.

The recovery of androsterone from both components of the original partition was good and its identification unquestionable. When the radioactive areas corresponded to authentic androsterone glucuronide or androsterone sulphate, hydrolysis always yielded at least androsterone. These two radioactive conjugates behaved in all systems as expected, in accord with the authentic controls. Selective hydrolysis confirmed these impressions. At first it was difficult to accept the observation that certain of the analytical procedures, such as countercurrent distribution, would give a group separation that contained both classes of conjugates. However, with the demonstration of the many metabolites of androsterone and their

marked differences in polarity on being chromatographed, it was easy to imagine that their conjugates would not fall into the simple two-component system predicted for the conjugates of androsterone itself.

Quantitative results could best be obtained from the paper-chromatography system for simple glucuronides. This system clearly separated the two classes of conjugates into two areas without cross-contamination. Therefore the kidney actively forms both classes of conjugates *in vitro*, with the sulphate system predominant when the substrate is presented in tracer quantities. This generalization does not imply that each of the free-steroid metabolites was also equally conjugated into two classes, since analysis of this was impossible. No glucuronides could be isolated from the anaerobic incubations; however, the sulphation process remained intact. The amount of steroid glucuronide formed represents about 3-4% of the steroid supplied and this same percentage conversion would probably hold true for each individual steroid, androsterone being the only one adequately followed to draw this conclusion with any degree of certainty.

Comparison of the findings for glucuronide formation with other experiments is subject to numerous qualifications. The most comprehensive studies of glucuronyltransferase used acceptors other than steroids, such as *o*-aminophenol and 2,4-dinitrophenol (Isselbacher, Chrabas & Quinn, 1962), as well as a variety of other acceptors to demonstrate ester, ether and other types of linkages in glucuronide formation. The studies *in vivo* are in disagreement (Cohn & Hume, 1960), but here there was a species difference. It is therefore considered that, before conclusions can be derived, comparative studies should be done.

Certain difficulties arise when sulphation is considered. Previous attempts to demonstrate that the kidney has an active sulphation system have been unsuccessful (Nose & Lipmann, 1958). Perhaps this failure on the part of previous investigators results from failure to use the proper acceptors.

These experiments differ from others previously reported in a number of details whose significance is unknown. No solubilizing agents were used (such as methanol and propylene glycol), so that the problem of their influence in the metabolic pathways is avoided. The small amount of steroid employed/g. of tissue allowed the experiment to go to equilibrium, and the results are believed to represent this state. In contrast, high substrate loads may yield a different pattern of products. Results gathered in such a fashion may completely obscure subtle but physiologically important activity. This probably accounts for the failure of other investigators who have specifically looked for

certain metabolic pathways such as sulphation in the kidney. It is also noteworthy that no fortifying factors were necessary for glucuronide and sulphate conjugate formation. Attempts were made to stimulate both systems with a variety of energy-active intermediates, but stimulation was never demonstrated (Pogell & Leloir, 1961). It can be concluded that within the kidney slice enough of the metabolic intermediates are present to handle the amount of acceptor added in the biosynthetic pathways, or else that the cofactors failed to enter the cell and reach the site of the conjugation.

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