STUDIES ON THE IN VITRO PERFUSION OF STEROIDS THROUGH THE DOG KIDNEY*

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One of the earliest records on the perfusion of the dog kidney dates back to that of Ludwig in 1863 (1) in which he attempted to study the mechanical factors functional in a dead organ. Since then, a number of investigators have used perfusion techniques in order to elucidate the process or processes by which urine is formed. The early literature on kidney perfusions has been reviewed (2).

Although the metabolism of steroid substances on perfusion through the mammalian adrenal has been extensively investigated (3), knowledge of the rôle of the kidney in the metabolism and excretion of steroid substances is lacking. It is well known that urinary steroid metabolites occur for the most part in conjugated form, either as glucuronides, sulfates, or some other as yet unidentified form. The evidence on this point has been summarized elsewhere (4).

The primary aim of the study presented in this paper was to determine whether or not the mammalian kidney conjugates steroid substances. Both effluent renal blood and urine excreted during vascular perfusion were studied for evidence of conjugation and other metabolic changes in steroids added to the perfusate.

A series of perfusion experiments upon dog kidneys with dehydroepiandrosterone, testosterone, 17α-hydroxyprogesterone, Reichstein’s Compound S, cortisol, hydrocortisone, and corticosterone added to the perfusion fluid was completed. The perfusates were extracted with ethyl acetate, and the urines excreted during perfusion were fractionated into the free and into the sulfate- and glucuronide-conjugated fractions. These extracts were subjected to extensive chromatography on paper and silica gel.

EXPERIMENTAL

Mongrel dogs were anesthetized intravenously with Nembutal sodium, 1 ml. (60 mg.) per 5 pounds of body weight. Upon opening the abdomen,

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Fractionation of Steroid Components of Urine into Free, Sulfate, and Glucuronide Fractions

Urine
(1) Adjusted to pH 7.0
(2) Extracted 4 times with equal volume ether-chloroform (4:1)

Ether-chloroform
(1) Washed 3 times with 0.05 volume cold 0.1 N NaOH
(2) Washed 3 times with 0.05 volume distilled H₂O
(1) Dried over anhydrous Na₂SO₄
discarded
(2) Concentrated to dryness under vacuum

Washings

Ether-chloroform
(1) Washed 3 times with 0.05 volume cold 0.1 N NaOH
(2) Washed 3 times with 0.05 volume distilled H₂O
(1) Adjusted to pH 7.5-3.0
(2) Extracted 5 times with equal volume of n-butyl alcohol

Butyl alcohol extract
(1) Washed 4 times with 0.1 volume distilled H₂O
discarded
(2) Concentrated to dryness under vacuum

Butyl alcohol residue
(1) Dissolved in 20-30 ml. dioxane containing 10% trichloroacetic acid
(2) Heated 10 min. in boiling water bath

Dioxane solution
(1) Adjusted to pH 7.0 with NaOH
(2) Concentrated to dryness under vacuum

Dioxane residue
(1) Dissolved in 100 ml. distilled H₂O
(2) Extracted 4 times with equal volume ether-chloroform (4:1)
H₂O layer

(1) Removed traces of organic solvents by bubbling \( \text{N} \) through solution under vacuum
(2) Added 10 ml. 0.2 \( \text{M} \) NaAc and adjusted to pH 4.8 with 0.3 \( \text{M} \) HAc
(3) Added 50,000 units penicillin, 100,000 units \( \beta \)-glucuronidase,* incubated 24 hrs.
(4) Repeated step (3)
(5) Adjusted pH to 1.0 with \( 6 \text{N} \) H₂SO₄
(6) Extracted 4 times with equal volume of ether-chloroform (4:1)

Ether-chloroform layer

(1) Washed 3 times with 0.05 volume cold 0.1 \( \text{N} \) NaOH
(2) Washed 3 times with 0.05 volume distilled \( \text{H}_2\text{O} \)

Washings Ether-chloroform discarded

(1) Dried over anhydrous \( \text{Na}_2\text{SO}_4 \)
(2) Concentrated to dryness under vacuum

Steroids previously conjugated as sulfates

* Ketodase, obtained from the Warner-Chilcott Laboratories.
careful dissection was undertaken to preserve sufficiently long segments of the renal artery and ureter to permit cannulation. Each dog was then heparinized by the injection of 10 ml. of heparin sodium, U. S. P., 5000 units per ml. (50 mg.), into the inferior vena cava. After heparinization, the right kidney was immediately removed, and the blood was then withdrawn from the aorta into a sterile evacuated plasma flask. The left kidney was removed while the heparinized blood was being collected from the aorta. The renal artery of each kidney was cannulated with a glass cannula and connected to a perfusion apparatus of the heart-lung type. The dog’s own heparinized aortic blood was diluted with an equal volume of a solution containing approximately 1 part of normal saline, 1 part of 5 per cent Amigen, and 1 part of 5 per cent dextrose. 1 gm. of streptomycin and 1,000,000 units of Penicillin were added to this perfusion fluid. Perfusion was immediately begun, and the ureters were cannulated with fine plastic tubing. The steroid substance for each experiment, in 20 ml. of propylene glycol, 2.5 ml. of benzyl alcohol, and 0.5 ml. of Tween 80, was made up to 100 ml. of final volume with 5 per cent dextrose and added at the site of cannulation of the renal arteries to drip slowly for the period of 2½ to 3 hours required by the experiment. The temperature of the kidneys and of the perfusion medium was maintained at 39.0° by a constant temperature bath from which the water was circulated by a pump to a water-jacketed perfusion chamber and to a coiled condenser supplying arterial blood to the kidneys. The blood volume was kept constant by the addition of saline to replace the urine excreted. The perfusion medium was kept well oxygenated by supplying gas containing 95 per cent O₂ and 5 per cent CO₂ filtered through physiological saline. The kidneys were perfused by gravity flow. The position of the blood reservoir was adjusted to produce an arterial pressure equivalent to 100 mm. of mercury as measured by a mercury manometer incorporated into the system.

During the experiments, urea in the blood was determined by the method of Karr (5) and in the urine by the titration of NH₃ liberated by the enzyme urease (6). Creatinine was estimated by the procedure of Folin and Wu (7). Sodium and potassium were estimated by flame photometric analysis.

The blood perfusates were hemolyzed by freezing and thawing, and the steroids were extracted by jetting the blood several times in a very fine stream through 1 liter of ethyl acetate (8). Whenever emulsions resulted, they were broken by centrifugation in the cold. The process was repeated with a second liter of ethyl acetate. Combined ethyl acetate extracts were washed three times with 0.1 volume of cold 0.1 N sodium hydroxide and three times with 0.1 volume of distilled water. The washed extracts were dried over anhydrous sodium sulfate and evaporated to dryness under
vacuum. The residues were partitioned between 70 per cent methanol and n-hexane to remove fatty material. The methanol fraction was evaporated to dryness under a fine stream of air in a water bath at 45°. These extracts were then subjected to further purification by paper chromatography in the appropriate solvent system by the methods of Burton et al. (9) as modified by Romanoff et al. (10). Strip widths were determined by the steroid content of the extract, as estimated by colorimetric reactions such as the Zimmermann reaction (11) for 17-ketosteroids and the blue tetrazolium (12) and formaldehydogenic reactions (13) for corticosteroids. The techniques for the detection of steroid substances on paper chromatograms, quantitative elution, and estimation in the ultraviolet have been discussed elsewhere (14). Sulfuric acid chromogens (15) were made on all eluted substances. Melting point determinations were made on a Fisher melting point block and are uncorrected. All infra-red analyses were made by the potassium bromide pressed disk technique upon a Beckman IR-2T single beam instrument within the region from 15.0 to 2.5 μ.

The urines excreted during perfusion were fractionated into the free and into the sulfate- and glucuronide-conjugated fractions by a modification of the dioxane-trichloroacetic acid procedure of Cohen and Oneson (16) for the hydrolysis of steroid sulfates. The procedure for the extraction of steroids from the urine and fractionation of these extracts into various groups is described in the accompanying diagram. All solvents were re-distilled, and the dioxane was purified according to Cohen and Oneson (16). The free, sulfate- and glucuronide-conjugated fractions obtained were subjected to further purification and fractionation by paper chromatography. Methods for the chromatography, detection, estimation, and identification of compounds were similar to those employed for blood extracts and are described above.

Results

In Table I are presented data from an experiment which was designed to test the viability and physiological function of the kidney preparation used in these experiments. The data were obtained upon one kidney which began to excrete urine 10 minutes after perfusion was begun. Urea and creatinine were added to the perfusate to bring the concentration of these substances above the normal levels. The blood volume was kept constant by replacing the volume of urine formed with an isosmotic saline. Urine and blood samples were collected at 10 minute intervals. It can be seen that the rate of excretion of urine was fairly constant throughout the experiment and agrees fairly well with the rates reported by Starling and Verney (2) at approximately the same arterial pressure. The specific gravity of
the urine is close to normal at the beginning of the experiment but declined somewhat towards the end. The levels of sodium in the urine are much lower than those in blood, while the reverse is true for potassium. This indicates that sodium is being reabsorbed from the glomerular filtrate by the tubules, while blood potassium is being secreted by the tubules. It is also apparent that the concentrations of both urea and creatinine are always higher in the urine than in the blood. This again indicates concentration and excretion of these substances by the kidney. The data indicate that the kidney preparation employed in this in vitro perfusion experiment is functional.

**Table I**

*Data on in Vitro Perfusion of Dog Kidney*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Secretion</th>
<th>Specific</th>
<th>Na</th>
<th>K</th>
<th>Urea</th>
<th>Creatinine</th>
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<td>No.*</td>
<td>rate ml. per min.</td>
<td>gravity,</td>
<td>eq.</td>
<td>eq.</td>
<td>mg. per</td>
<td>mg. per</td>
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<td>84.4</td>
<td>15.3</td>
<td>7.0</td>
<td>0.38</td>
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<td>77.3</td>
<td>16.4</td>
<td>7.9</td>
<td>0.38</td>
</tr>
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<td>1.7</td>
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<td>71.3</td>
<td>17.4</td>
<td>7.0</td>
<td>0.35</td>
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<tr>
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<td>1.9</td>
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<td>71.3</td>
<td>16.6</td>
<td>6.6</td>
<td>0.33</td>
</tr>
<tr>
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<td>75.6</td>
<td>15.3</td>
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<td>0.31</td>
</tr>
<tr>
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<td>75.8</td>
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<td>6.0</td>
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<td>81.9</td>
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<td>86.0</td>
<td>12.7</td>
<td>4.9</td>
<td>0.23</td>
</tr>
<tr>
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<td>1.007</td>
<td>88.8</td>
<td>12.7</td>
<td>4.9</td>
<td>0.23</td>
</tr>
</tbody>
</table>

* Collected at 10 minute intervals.

Fifteen perfusion experiments were completed: three with hydrocortisone, two with cortisone, two with Reichstein's Compound S, two with 17α-hydroxyprogesterone, two with dehydroepiandrosterone, one with testosterone, and three controls without added steroid. Two kidneys from the same dog were used in each perfusion experiment. The amount of blood taken from each dog to prepare the perfusion medium averaged 360 ml. In general the kidneys began to make urine 10 to 15 minutes after perfusion was begun. The average perfusion time was 3 hours, the urinary excretion rate was 1.4 ml. per minute, and the perfusion rate through the kidneys was 78 ml. per minute. The creatinine (17) concentration in the urine averaged 25 mg. per cent.

Both blood and urinary extracts were put through a series of chromatographic systems suitable for the most polar to the least polar steroids (10)
to eliminate the possibility that metabolites of the perfused compound might escape detection.

**Perfusion of Kendall’s Compound F**

On the basis of formaldehydogenic determinations (13) and the blue tetrazolium (BTZ) reactions (12), the extracts from the first blood perfusate contained 29.7 mg. of α-ketolic substance; hydrocortisone was used as a reference standard. After chromatography in toluene saturated with propylene glycol for a 48 hour period (with several 15 cm. wide strips), a compound with the mobility of that of hydrocortisone was detected. It absorbed in the ultraviolet and gave a positive reaction to blue tetrazolium. After elution and recrystallization, 27.6 mg. of crystals having an absorption maximum in methanol at 242 μ were obtained. The sulfuric acid chromogen (15) absorbing from 220 to 600 μ was identical with that of crystalline hydrocortisone, with maxima at 240, 280, 390, and 475 μ. The infra-red spectrum was identical with that of hydrocortisone. The substance melted at 208–209°. A mixture with standard hydrocortisone, 206–208°, melted at 206–208°.

Several trace substances, all of which gave a positive reaction to the BTZ reagent, were located in the blood perfusate. One was more polar than Compound F, and several were less polar. On the basis of sulfuric acid chromogens and infra-red data, it was concluded that these substances were non-steroidal contaminants.

On fractionating the urine, it was found that the free fraction contained 20.7 mg. of α-ketolic substance on the basis of BTZ (12) and formaldehydogenic (13) reactions. After chromatography, 11.9 mg. of crystalline hydrocortisone were obtained. It had an absorption maximum in methanol at 242 μ. From appraisal of the sulfuric acid chromogen, infra-red data, and melting point determinations, it was considered identical with standard hydrocortisone. Steroids more polar or less polar than hydrocortisone could not be located in the free fraction.

The sulfate and glucuronide fractions of the urine contained no steroidal substances on the basis of the BTZ and formaldehydogenic reactions. This was confirmed by paper chromatography with the use of all solvent systems (10) suitable for the most polar to the least polar steroids.

The second perfusion experiment yielded approximately the same results. The BTZ and formaldehydogenic reactions indicated the presence of 34.8 mg. of α-ketol in the perfusate and 15.3 mg. in the free fraction of the urine. After chromatography and recrystallization, 31.2 mg. and 9.2 mg. respectively of crystalline hydrocortisone were obtained from the extracts. No steroids were found in the sulfate or glucuronide fractions of the urine, and this was verified by chromatographic studies. The third perfusion produced approximately the same results.
In all these experiments approximately 40 per cent of the starting material was recovered as crystalline hydrocortisone. In the search for additional steroids, the kidneys from the last perfusion were homogenized and extracted for steroid substances. After partitioning between 70 per cent methanol and n-hexane, this extract was chromatographed in the same system employed for the blood perfusates. The only steroid compound isolated and identified was hydrocortisone, 4.5 mg., which melted at 209-210°. There was no depression of the melting point on admixture with the starting material. The infra-red spectrum was also identical with that of the starting material.

A substance more polar than hydrocortisone was also isolated from the kidney. It did not have any maximum in methanol in the ultraviolet region but gave a positive reaction to the BTZ reagent. It gave maxima in concentrated sulfuric acid (15) at 262 and 322 μ, the latter peak having a much higher extinction coefficient. The identity of this substance has not been established. No other steroid substances could be detected.

Perfusion of Kendall’s Compound E

Two perfusion experiments with 100 mg. of cortisone were completed. On the basis of BTZ and formaldehydogenic reactions, the perfusate contained 20.0 mg. of α-ketolic substance, while the free fraction of the urine contained 11.5 mg. Chromatography followed by recrystallization yielded 14.0 mg. of cortisone in the perfusate and 8.7 mg. in the free fraction of the urine. The identification was based on chromatographic behavior, sulfuric acid chromogens, infra-red spectra, and melting point and mixed melting point determinations with the starting material.

Several spots were located on paper, both more polar and less polar than cortisone, each of which gave the BTZ reaction and absorbed in the ultraviolet. However, these spots proved to be contaminants as judged from infra-red data. No cortisone or any other steroid was found in the sulfate and glucuronide fractions of the urine. The second perfusion experiment with cortisone yielded essentially the same results.

Perfusion of Reichstein’s Compound S

In each of two experiments, 100 mg. of Compound S were perfused through the kidneys. The results were similar to the hydrocortisone perfusions except that the recovery of Compound S from the free fraction of the urine was lower. Chromatography followed by recrystallization yielded 32.5 mg. of crystalline Compound S from the perfusate and only 2.9 mg. from the free fraction of the urine. The identification was based on chemical and physical criteria described for the previous compounds. Again no steroidal substances were found in the sulfate and glucuronide fractions of the urine. The second experiment produced the same results.
Perfusion of 17α-Hydroxyprogesterone—The two perfusions with 100 mg. each of 17α-hydroxyprogesterone yielded somewhat different results. The recovery of the starting material from the perfusate was much lower than that found in the previous experiments with other compounds. The quantities found in the free fraction of the urine were even much less, on the order of 0.5 per cent. After chromatography and recrystallization from methanol, the perfusates from the two experiments yielded an average of 8.8 mg. of crystalline 17α-hydroxyprogesterone. Its sulfuric acid chromogen and infra-red spectrum were identical with those of the starting material, and it melted at 210–212°. On admixture with the starting material, m.p. 213–216°, the mixture melted at 213–215°. No metabolites of the starting material were found.

The free fraction of the urine in both experiments yielded an average of 453 γ of 17α-hydroxyprogesterone which was estimated from its ultraviolet extinction coefficient in methanol at 242 mμ. It was identified on the basis of its mobility on paper, its sulfuric acid chromogen with maxima at 290 and 430 mμ, and its infra-red spectrum. Again, no steroidal substances were found in the sulfate and glucuronide fractions of the urine.

Perfusion of Dehydroepiandrosterone—The perfusion of 100 mg. of dehydroepiandrosterone in each of two experiments yielded approximately the same results as were obtained with 17α-hydroxyprogesterone. After chromatography and recrystallization from methanol, an average yield of 9.3 mg. of dehydroepiandrosterone was obtained from the two perfusates. The substance melted at 146–148°, and on admixture with the starting material, m.p. 148.5–149.5°, the mixture melted at 147–148°. Its sulfuric acid chromogen and infra-red spectrum were identical with those of the starting material.

The free fraction of the urine yielded an average of 250 γ of material which was tentatively identified as dehydroepiandrosterone from its mobility on paper and its sulfuric acid chromogen (15) with maxima at 306 and 410 mμ. No steroidal substances were found in the sulfate or glucuronide fractions.

Perfusion of Testosterone

One perfusion was completed with 100 mg. of testosterone. Chromatography of the perfusate revealed the presence of two substances, one with the mobility of testosterone and the other with the mobility of Δ4-androstene-3,17-dione. These substances were eluted separately and further purified by chromatography on silica gel. The samples were dissolved in benzene-hexane (1:1) and chromatographed on silica gel. Elution was begun with benzene-ether (19:1) with a gradual increase in the concentration of ether.

The sample with the lower mobility yielded 9.8 mg. of testosterone with
PERFUSION OF STEROIDS

a melting point of 150–152° which was not altered on admixture with the starting material, m.p. 152.5–154.0°. Its infra-red spectrum was identical with that of the starting material.

The sample with the higher mobility yielded 6.3 mg. of crystalline material which melted at 170–171°. On admixture with standard \( \Delta^4 \)-androstene-3,17-dione, m.p. 170.5–171.5°, there was no depression of the melting point. The infra-red spectrum contained a \( \text{C=C} \) carbonyl band at 5.76 \( \mu \), an \( \alpha,\beta \)-unsaturated carbonyl band at 6.02 and 6.16 \( \mu \), and finger-print bands identical with those of \( \Delta^4 \)-androstene-3,17-dione.

The free fraction of the urine yielded 1.0 mg. of a substance with an absorption maximum in methanol at 240 mp. Its infra-red spectrum showed a carbonyl band at 5.78 \( \mu \), an \( \alpha,\beta \)-unsaturated carbonyl band at 6.02 and 6.16 \( \mu \), and finger-print bands identical with those of \( \Delta^4 \)-androstene-3,17-dione. Testosterone was not found in the free fraction of the urine. Again, no steroidal substances were found in the sulfate and glucuronide fractions of the urine.

DISCUSSION

The in vitro perfusion technique has been employed in experiments to study the action of the kidney on steroid substances. Evidence was presented to show that a good physiological kidney preparation was employed. The perfused kidney was capable of sodium retention and at the same time was functional in the concentration and excretion of urea and creatinine and in the secretion of potassium.

It has been shown that the dog kidney does not conjugate steroids either as sulfates or glucuronides under the experimental conditions described here. The data demonstrating the viability and physiological function of the kidney preparation make it highly unlikely that the renal "conjugating capacity" may have been impaired. It is probable that a hydrolytic equilibrium between the free and conjugated forms is not involved because it would result in the finding of both conjugated and free steroids in the urine. From these experiments the free form only was recovered in both urine and blood.

In general, the recovery of the starting steroid material in crystalline form from the perfusate was much less in experiments with the less polar steroids such as 17\( \alpha \)-hydroxyprogesterone, dehydroepiandrosterone, and testosterone than with the more polar steroids such as hydrocortisone, cortisone, and Compound S. This may be explained by the fact that all the blood extracts were partitioned between 70 per cent methanol and \( n \)-hexane. The loss of the less polar steroids into the \( n \)-hexane fraction is appreciable. However, this partitioning was essential in order to remove the large quantities of the fatty substances which accompanied an ethyl acetate extraction of blood.
Approximately 9 to 12 mg. of crystalline substance were recovered from the free fraction of the urine in the case of the cortisone and hydrocortisone perfusions. In the case of the perfusion with 17α-hydroxyprogesterone and dehydroepiandrosterone, only 453 and 250 γ respectively of the starting material were recovered. Two factors may be functional: one is the water solubility of steroids and the other is the protein-binding capacity of the steroids. On the basis of their solubility studies in buffer and protein solutions (bovine serum albumin), Eik-Nes et al. (18) have divided a number of steroids into several groups. Cortisone is representative of the first group which has a high water solubility with poor protein-binding capacity. Dehydroepiandrosterone, testosterone, and Δ4-androstene-3,17-dione fall into the second group which possesses poor to fair water solubility with moderate protein-binding. A combination of these two factors, water solubility and protein-binding capacity, may have been functional in these perfusion experiments to yield relatively large quantities of the more polar steroids and minute quantities of the less polar steroids in the free fraction of the urine.

The perfusate from the testosterone experiment yielded both the starting material (9.8 mg.) and Δ4-androstene-3,17-dione (6.3 mg.). The amount of the latter compound isolated was approximately 60 per cent of the weight of starting material isolated. The interconversion of testosterone and Δ4-androstene-3,17-dione has been demonstrated in both rabbit liver and kidney homogenates by Kochakian and his associates (19–22). The same conversion in rabbit kidney mince was reported by West and Samuels (23). An important factor to be considered, however, is the finding by Richterich-van Baerle et al. (24) that human serum can convert testosterone to Δ4-androstene-3,17-dione. This is under investigation in our laboratories.

It was interesting to note, however, that the free fraction of the urine yielded 1.0 mg. of Δ4-androstene-3,17-dione but no testosterone. Since both substances fall into the same group as far as water solubility and protein-binding are concerned (18), one would expect to find both substances in the free fraction of the urine. However, since both are not found, some additional factor may require the conversion of testosterone into Δ4-androstene-3,17-dione before excretion by the kidney results. This explanation is supported by the fact that testosterone has been isolated from urine in trace amounts only after the parenteral administration of that substance in large amounts (25).

SUMMARY

A technique was developed for the in vitro perfusion of a functional kidney preparation capable of excreting urine at the average rate of 1.4 ml. per minute. A number of perfusion experiments with the following
steroids were completed: hydrocortisone, cortisone, Reichstein's Compound S, 17α-hydroxyprogesterone, dehydroepiandrosterone, and testosterone. The urine was fractionated into the free, sulfate, and glucuronide fractions, and the perfusates were extracted with ethyl acetate. Separations and purifications were effected by paper and silica gel chromatography. It was shown that 9 to 12 per cent of crystalline steroid was recovered in the free fraction of the urine in the case of the cortisone and hydrocortisone perfusions and 2.9 per cent in the Compound S perfusions. However, in perfusions with less polar steroids, only microgram quantities were recovered in the free fraction of the urine; the possible rôle of water solubility and protein-binding capacity of steroids in this regard is suggested. No metabolites of the perfused steroids could be isolated in the perfusion with cortisone, hydrocortisone, Reichstein's Compound S, 17α-hydroxyprogesterone, and dehydroepiandrosterone in either the perfusates or the urine. The perfusion with testosterone yielded 9.8 mg. of testosterone and 6.3 mg. of Δ4-androstene-3,17-dione in the perfusate. The free fraction of the urine yielded 1.0 mg. of Δ4-androstene-3,17-dione and no testosterone. In none of the perfusion experiments were any steroids found in the sulfate or glucuronide fractions of the urine.

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