CHEMICAL TRANSFORMATIONS OF STEROIDS BY ADRENAL PERFUSION

V. BLOOD INCUBATIONS AND PERFUSIONS WITH CORTISONE*

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The importance of cortisone as a therapeutic agent has stimulated the extension of investigations into the various facets of its fate in the animal body. It was of interest for instance to learn whether further transformations, if any, of this steroid occurred at the site of its synthesis. The development of the adrenal perfusion technique in these laboratories (1) made this means a convenient tool for this study. Thus cortisone was perfused by Dr. C. G. Bergstrom* through isolated bovine adrenal glands as the first steroid containing an oxygen function at carbon atom 11. From the perfusate, subjected to the usual charcoal procedure (2), 28 per cent cortisone and 2 per cent adrenosterone were crystallized. This unusually low yield in total crystalline material made further investigation necessary.

Duplication of the perfusion yielded similar results. Not only was adrenosterone isolated from the perfusate, but also in experiments in which cortisone was circulated in the perfusion medium (whole citrated blood) alone or was subjected to charcoal adsorption simply from an aqueous solution. In every instance the recovery of cortisone was poor. It thus appeared that the charcoal procedure was inadequate for this investigation and a search for other means of extraction was undertaken.

Three general methods were known for the extraction of corticosteroids from blood without the prior precipitation of the proteins. The charcoal adsorption procedure (2) has been used extensively in the extraction of perfusates. It has the advantage in that only relatively small liquid volumes are involved. The recoveries of steroids with this procedure did not always prove satisfactory (3), and the possibility of artifact formation on the charcoal surfaces was considered (2). However, these disadvantages do not invalidate most of the results obtained in previous work with use of this technique.

A second method consisted of dialysis of the perfusate against aqueous media (4). Although the extraction of the dialysate with an organic sol-

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1 Present address, G. D. Searle and Company, Chicago 80, Illinois.
vent yielded relatively pure extracts, the lengthy periods required for dialysis were undesirable. Moreover, application on a large scale was found to be inconvenient (2). Dialysis against a two-phase system, saline-charcoal (2) and more recently aqueous methanol-chloroform (5), has also been described.

Finally, the direct extraction of corticosteroids with organic solvents has the advantage of a rapid extraction. The treatment of blood, particularly when diluted, with chloroform (4) and ether (6) has led to troublesome emulsions, necessitating separation by centrifugation. This difficulty was generally not encountered with a chloroform-ether mixture (1:4) when the blood was only slightly diluted (3). A further development of the direct extraction method seemed to show the most promise for our needs.

Various solvents recognized for their favorable solubility properties for corticosteroids (7) were tested for use with blood. Those forming emulsions during manipulation were eliminated. Particularly favorable was ethyl acetate, since it extracted only one-half the amount of extraneous material compared to that obtained with the ether-chloroform mixture. Moreover, maximal recoveries of cortisone (90 per cent) could be achieved with smaller volumes of solvent. Considerable improvement over the charcoal procedure thus was seen in the recovery of this steroid from blood. In addition, no formation of adrenosterone was evident, providing further proof that the relatively large quantities of adrenosterone found previously were an artifact of the charcoal treatment. A study of the biochemical transformations of cortisone could now be undertaken.

In the incubation of cortisone with bovine blood an approximate 11 per cent decrease (based on control recoveries) in material containing the α,β-unsaturated ketonic grouping was noted. This diminution could be, in the main, accounted for in one or more reduced products with the intact ketol side chain. This material moved on paper chromatograms at a rate comparable to that of tetrahydrocortisones (3-OH,5-H trans). The area occupied by this substance (or substances) is thus referred to as the "tetrahydro zone." No products moving at a rate of dihydrocortisone could be detected.

In two perfusions of cortisone through adrenals, the disappearance of unsaturated ketonic material was in the range of 29 per cent. However, the quantity of products found in the tetrahydro zone was not more than 10 per cent. In contrast to the blood incubations, these perfusions were conducted with larger amounts of starting material to permit isolation of products formed. From the tetrahydro fraction a small amount of tetrahydrocortisone (3β,allo) (allopregnane-3β,17α,21-triol-11,20-dione) was separated and identified. An additional substance, alloidihydrocortisone (allopregnane-17α,21-diol-3,11,20-trione) was isolated in about a 1 per
cent yield. Minute quantities of adrenosterone were also found. Trace amounts of other transformation products were detected in paper chromatogram analyses. Crystalline cortisone was readily recovered.

The occurrence of adrenosterone in the adrenal perfusate is not easily interpreted for the time being, since it was found that the side chain of cortisone is degraded under relatively mild conditions. Adrenosterone was demonstrated to be formed from cortisone through charcoal treatment, followed by elution with low boiling solvents without exclusion of the access of air. Ether peroxides and exposure of cortisone solutions to elevated temperatures (95°) produced the same degradation.

Methods

Steroids—The steroids were chromatographed on silica gel (Davison, No. 923, 100 to 200 mesh) and crystallized to a constant melting point before use. The melting points were observed on a Fisher-Johns hot-stage through a microscope and are corrected (accuracy ±2°). The infra-red analyses were made with a single beam Perkin-Elmer infra-red spectrometer (model 12C). The molar absorption coefficient (ε) was determined at λ<sub>max</sub> 238 μ (methanol) on a Beckman spectrophotometer (model DU) according to the formula ε = (D/(l × c)), where D is the optical density, l the light path in cm., and c the concentration in moles per liter.

Cortisone was obtained from its acetate (Glidden) after hydrolysis with

2 The infra-red analyses and interpretations of Dr. H. Rosenkrantz and Mr. L. Zablow are gratefully acknowledged.
potassium bicarbonate (8) under nitrogen. It melted after crystallization from acetone at 216–224° and showed \([\alpha]_D^{28} +215° \pm 3° (c, 0.524 \text{ in } 95\text{ per cent alcohol}).\) \(\epsilon\) had the same value \((1.52 \times 10^4)\) at 237 to 239 m\(\mu\) when cortisone was dissolved in methanol and at 243 m\(\mu\) when dissolved in chloroform. Mattox and Kendall (9) obtained cortisone from its acetate by acid hydrolysis, having a melting point of 230–231° and \([\alpha]_D^{24} +212° (\text{alcohol}).\)

Adrenosterone was prepared by chromic acid oxidation of cortisone (10) according to the procedure described in the next paragraph. It had after crystallization from methanol a melting point of 223–225°. \([\alpha]_D^{20} +306° \pm 3° (c, 1.090 \text{ in chloroform}), \text{ or } [\alpha]_D^{21} +270.5° \pm 4° (c, 1.044 \text{ in acetone})\) and \(\epsilon 1.62 \times 10^4\) were observed. As a mineral oil mull, it showed absorption bands near 1748 (carbonyl at C\(\equiv\)), 1707 (carbonyl at C\(\equiv\)), 1668 and 1600 (unsaturated carbonyl at C\(\equiv\)), 1224, 1092, 1051, 1014, and 876 cm\(^{-1}\) (some finger-print bands). Newer constants for adrenosterone have been reported recently by Herzog et al. (11): m.p. 218.5–220°, \([\alpha]_D^{28} +271° (\text{acetone}), \epsilon_{338} 1.51 \times 10^4 (\text{alcohol}).\)

Preparation of Adrenosterone—1.5 gm. of cortisone were dissolved in 90 ml. of glacial acetic acid (distilled over chromium trioxide). To this solution 830 mg. of chromium trioxide in 1 ml. of water and 10 ml. of glacial acetic acid were added. After standing for 16 hours at 25°, the reaction mixture was cooled to 0° and shaken with 1 gm. of sodium sulfite. 200 ml. of ice water were then added, and the solution neutralized in the cold with 5 N sodium hydroxide. It was subsequently extracted in portions of 100 ml. with 2 \(\times\) 250 ml. of benzene. The extracts were washed consecutively with 2 N sulfuric acid, water, 2 N sodium carbonate, and water. After drying over sodium sulfate, the solutions were combined and evaporated \(\text{in vacuo} at 35°.\) 1.05 gm. \((85 \text{ per cent of theoretical})\) of crude product, m.p. 200–225°, were obtained. Two crystallizations from acetone-ether yielded 0.95 gm. of adrenosterone melting at 222–225°, \([\alpha]_D^{25} +301° \pm 4° (\text{chloroform}).\)

Blood—Blood was collected from the severed neck vessels of stunned cattle or in three cases drawn aseptically by puncture of the jugular vein. Approximately 3.4 liters were placed in bottles containing 600 ml. of an acid citrate-glucose solution with addition of 0.5 gm. of terramycin² or 100,000 units of penicillin G and 0.5 gm. of streptomycin. The blood was kept at 4° and used within 24 hours.

Incubation Experiments—40 ml. of blood were added to 250 ml. Erlen-

³ The author is indebted to C. A. Fish and J. Hedin for the collection of the blood. The aseptic samples were drawn under supervision of Dr. A. Macen.

⁴ The terramycin was obtained as a gift from Dr. H. Seneca, Chas. Pfizer and Company, Inc.
meyer flasks containing 4 mg. of steroids in 0.25 ml. of propylene glycol. The mixture was shaken under an O₂-CO₂ atmosphere at 38° for 3 hours. These are designated as blood incubations. Blood blanks with propylene glycol alone were run in parallel. In recovery experiments the blood (cooled to 0°) was added to the steroid solution after the incubation and immediately frozen.

Aliquots from two blood incubations were tested for the presence of bacteria by plating on a brain-heart infusion agar (Difco). No growth was noted.⁵

**General Method for Blood Extraction with Organic Solvents**—The incubated blood was rapidly frozen in an acetone-dry ice bath and stored over night at −6°. After thawing in a 40° bath, 6 gm. of sodium chloride were added to the hemolyzed blood to diminish the solubility of steroids. The blood was then transferred to a 200 ml. separatory funnel (Squibb) with the aid of 2 × 2 ml. of saturated aqueous sodium chloride and shaken vigorously for 6 seconds at 4° with 80 ml. of organic solvent.⁶ After standing 5 minutes at 4°, the solvent layer was syphoned off and the operation repeated with another portion of 80 ml. of solvent. No emulsification is encountered under these conditions. The two extracts were subsequently washed consecutively with 2 ml. portions of saturated sodium chloride until no further color was eluted (approximately five times). After standing over anhydrous sodium sulfate for 10 minutes, the combined extracts were evaporated to dryness in vacuo below 40°. The residue was dissolved in methylene chloride and filtered into a tube. It was then evaporated repeatedly with additions of benzene in a current of nitrogen to a constant weight. Volumes up to 150 ml. of incubated blood were treated in the manner described with comparable results.

**Analysis of Extracts**—The residue obtained above was redissolved in chloroform and the concentration of α,β-unsaturated ketonic material determined with the ultraviolet spectrophotometer.

Resolution of steroids was accomplished by the paper chromatography method of Zaffaroni et al. (12). The toluene-propylene glycol system (13) or the ligroin-propylene glycol modification of Savard (14) for less polar compounds was employed. Paper strips 57 cm. in length and of varying widths were impregnated, unless otherwise stated, with a 30 per cent methanolic solution of propylene glycol and immediately blotted. The development periods at approximately 30° were varied so that compounds within

⁵ The microbiological assay was carried out by Miss G. Weber, Worcester State Hospital.

⁶ Non-hemolyzed blood must be shaken for approximately 15 seconds in order to achieve total hemolysis and requires a larger ratio of solvents to prevent emulsification.
a wide polarity range should have been noted if present: with ligroin, 3 and 20 hours; with toluene, 6, 17, and 60 hours. Thereafter the compounds were located by scanning the paper under ultraviolet light (15) and with the use of various color tests: Zimmermann reagent, particularly for 17-ketosteroids (6), 2,4-dinitrophenylhydrazine for ketones, particularly when conjugated with an unsaturation (6), alkaline triphenyl tetrazolium solution (TPTZ) for reducing groups, especially the 20,21-ketols (13), and fuming sulfuric acid (16).

For quantitative estimations specific areas of duplicate strips were eluted with methanol, and the amount of steroids determined by ultraviolet absorption at 238 mp, or by the blue tetrazolium reaction of Chen and Tewell for the 20,21-ketol side chain (17).

Adrenal Perfusions of Cortisone—The two adrenal perfusions were carried out according to a procedure detailed elsewhere (1). Blood aseptically drawn from two animals and combined was employed. In Perfusion A, a solution of 473 mg. of cortisone in 20 ml. of propylene glycol was combined with 4.3 liters of blood. This mixture was circulated at 38° under an O₂-CO₂ atmosphere via venous entry through six lacerated bovine adrenals placed in parallel. Eight cycles requiring a total of 3 hours were run. In Perfusion B, 3.9 liters of blood containing 430 mg. of cortisone in 20 ml. of propylene glycol required 2 hours for the completion of eight cycles. 150 ml. aliquots of the perfusates were taken for comparison with incubation experiments carried out with the identical blood.

Extraction of Adrenal Perfusates—3.7 liters of Perfusate A, in which 555 gm. of sodium chloride (analytical reagent) had been dissolved, were shaken with 10 liters of ethyl acetate (analytical reagent) at 4° in a 20 liter Pyrex bottle for approximately 30 seconds. After standing for 5 minutes at 4°, by which time the phases had clearly separated, the solvent layer was syphoned off. The extraction was repeated with 8 liters of ethyl acetate. The solvent layers, washed and treated according to the general procedure outlined above, afforded 3.0 gm. of residue. This weight was 2.5 times that calculated from the results (49 mg.) of the 150 ml. aliquot extraction (above).

3.44 liters of Perfusate B plus 516 gm. of sodium chloride were divided into two parts and shaken consecutively with the same 7 liters of ethyl acetate. After separation the extraction was repeated twice with 6.5 liters of ethyl acetate. The residue was 5.3 gm. The 150 ml. aliquot yielded 123 mg. of dried material. The blood blank was also unusually high (see "Results").

The technique of Dr. R. C. Haynes, Jr., with Mineralight model SL 2437 was applied.

The adrenal perfusions were carried out by Mr. C. A. Fish.
The residue of 5.3 gm. was partitioned between equal volumes of hexane and 80 per cent aqueous methanol (70 ml.). After separation of the phases the methanol extract was diluted to 60 per cent and finally to 25 per cent for the second and third shakings with equal volumes of hexane. Each of the hexane fractions was then consecutively back-washed with aqueous methanol of appropriate concentration. The aqueous methanol extracts were combined and reduced in vacuo to a small volume, the remaining aqueous solution saturated with sodium chloride, and the steroids extracted with ethyl acetate. 797 mg. of residue were obtained.

The appearance of larger quantities of extraneous material in these large scale extractions of corticosteroids from blood indicates that the procedure has yet to be improved. Obviously the denaturation of blood proteins is more extensive under these conditions. Investigations on this problem are now in progress.

Preparation of Reference Compounds

Allodihydrocortisone Acetate (Allopregnane-17α,21-diol-3,11,20-trione-21-acetate)—9.3 mg. of allopregnane-3β,17α,21-triol-11,20-dione-21-acetate10 were dissolved in 0.22 ml. of glacial acetic acid and oxidized according to the procedure of Sarett (18) with 0.18 ml. of 80 per cent acetic acid containing 7.78 mg. of chromium trioxide. After 10 minutes of reaction at 25°C, the mixture was diluted with 4 ml. of water and extracted exhaustively with methylene chloride. The organic layers were washed with water, saturated aqueous potassium bicarbonate, and again with water, and yielded after evaporation of the solvent a crystalline residue of 11.3 mg. After two crystallizations from acetone-ether, 4.4 mg. of needles were obtained, m.p. 230–236°C. An infra-red analysis showed that this product was not identical with the starting material. A sample in the solid state showed bands near 3460 (hydroxyl), 1751, 1237, 1222 (acetate), 1739 (carbonyl at C₁₀), 1704 (carbonyls at C₃ and C₁₁), 1071, 1050, 1041, and 902 cm⁻¹ (some finger-print bands). Djerassi et al. (19) have reported for a product prepared by hydrogenation of cortisone acetate a melting point of 235–236°C and infra-red bands in the carbonyl region near 1750, 1736, 1716, 1706, and 1700 cm⁻¹ (in chloroform solution).

Tetrahydrocortisone-(3β,allo)-diacetate (Allopregnane-3β,17α,21-triol-11,20-dione-3,21-diacetate)—4.5 mg. of the 21-monoacetate were acetylated with pyridine-acetic anhydride in the usual manner. 5.4 mg. of solid afforded, after crystallization from acetone-ether, 2.5 mg. of crystals melting

9 A sample of tetrahydrocortisone-(3α,normal) partitioned in this manner, was recovered in quantitative yield and with an unchanged melting point.

10 The author wishes to express his gratitude to Dr. M. Tishler, Merck and Company, for this sample.
at 215–222.5°. Infra-red bands of a sample dissolved in carbon disulfide were found near 3460 (hydroxyls), 1748, 1262, 1239 (acetates), 1736 (carbonyl at C_v), 1710 (carbonyl at C_ii), 1106, 1045, 1032, and 976 cm.\(^{-1}\) (some finger-print bands). Reichstein et al. found for the diacetate of his Substance D isolated from beef adrenal extract a melting point of 223–224° (20). Jones et al. (21) have reported infra-red bands in the carbonyl region near 1746, 1724, and 1705 cm.\(^{-1}\) (in chloroform solution).

**TABLE I**

**Blood Extractions**

40 ml. of whole citrated blood were shaken twice for 6 seconds with 80 ml. of solvent. The values for the residues were compared with those from an ethyl acetate extraction run in parallel and corrected to the mean calculated from fourteen individual ethyl acetate extractions. The results of the absorption measurements at 243 m\(_\lambda\) are expressed in mg. equivalents of cortisone.

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Weight (mg.)</th>
<th>Mg. equivalents of cortisone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate</td>
<td>9</td>
<td>0.7</td>
</tr>
<tr>
<td>Ether-chloroform, 4:1</td>
<td>16</td>
<td>0.6</td>
</tr>
<tr>
<td>Ethyl acetate-chloroform, 3:1</td>
<td>13</td>
<td>0.9</td>
</tr>
<tr>
<td>Benzene</td>
<td>9</td>
<td>0.4</td>
</tr>
<tr>
<td>Butyl acetate</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Ether-dichloroethane, 2:1</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

**Results**

**Blood Extraction**—Results obtained in experiments in which direct extraction of incubated bovine blood with various solvents was carried out are presented in Table I. Ethyl acetate was found to be the most satisfactory solvent of those investigated in that the extracted material was lowest in weight. Benzene, while favorable in this aspect, was found to be less efficient in extracting cortisone. With isoamyl acetate and ethyl butyl ketone the separation of phases was inadequate.

Blood blank data from fourteen experiments showed that the residues from ethyl acetate extractions ranged in weight from 5.6 to 19.0 mg. (mean 9 mg.) per 100 ml. of blood and contained, on the basis of ultraviolet absorption measurements, 0.3 to 1.5 mg. equivalents (mean 0.7) of cortisone.\(^{11}\) Slightly lower values were observed with unincubated blood. On only one occasion, with a sample of blood aseptically drawn, were the values un-

\(^{11}\) This expression does not imply the presence of cortisone in blood blanks.
usually high, 33 mg. in weight and 4.7 mg. equivalents of cortisone. (This blood was used in Perfusion B.)

The mode of intermixture was found to be an important factor in the treatment of blood with solvents. For instance, when a blood sample was shaken for 2 minutes instead of the usual 6 seconds, the extract was more highly colored (yellow) and gave the following values: 19 mg. in weight and 0.9 mg. equivalent of cortisone for 100 ml. of blood. Less drastic treatment of blood, as may be possible for instance with the recently published percolation method for extraction of estrogens (22) and 17-keto-steroids (23) from urine, might lower the blank values while providing the maximal recovery of steroids.

Perfusion Blank—1.5 liters of the blood lot used for adrenal Perfusion A were recycled through two adrenal glands eight times without addition of steroids. A 150 ml. aliquot of this perfusate was extracted in the usual manner with ethyl acetate and gave the following values: 18 mg. of residue weight and 1.5 mg. equivalents of cortisone for 100 ml. of blood.

Recovery Experiments—Nine blood samples to which cortisone had been added in a concentration of 10 mg. per 100 ml. were extracted with ethyl acetate and showed a recovery of 88 ± 3 per cent as determined by optical density readings of the crude extracts or of the eluates of the cortisone zone of paper chromatograms. A further extraction with another portion of ethyl acetate yielded an additional 2 per cent. The same recovery percentages were also obtained in the following experiments: cortisone in concentration of 500 γ, adrenosterone in concentrations of 10 mg. and 500 γ per 100 ml. of blood (two experiments each).12

Two experiments with cortisone (10 mg. per 100 ml.), with ether-chloroform 3:1, gave a recovery of only 80 per cent. An additional 10 per cent was extractable with a third portion of this solvent mixture. Three portions of this mixture thus were necessary to achieve the same results as with two portions of ethyl acetate.

Blood Incubation and Adrenal Perfusion

Content of Unsaturated Ketonic Material—The quantities of α,β-unsaturated ketonic material recovered after blood incubation and adrenal perfusion with cortisone are shown in Table II. These figures are corrected for appropriate blanks run in parallel (see above). Aliquots of the extracts calculated as containing approximately 500 γ of steroids were also analyzed by paper chromatography. The same recovery percentages as

12 Hechter et al., using the ethyl acetate method, obtained approximately the same recovery with other corticosteroids including desoxycorticosterone, 17-hydroxyldesoxycorticosterone, and 17-hydroxyconicosterone when added to blood at a level of 10 mg. per 100 ml. (private communication).
those tabulated were found from measurements of the eluates of the cortisone zone. Therefore material with an $\alpha,\beta$-unsaturated ketonic grouping other than cortisone could only be present in minor quantities. The paper blank corrections amounted to 2 per cent for blood incubations and 4 per cent for adrenal perfusions.

Recoveries after two blood incubations of adrenosterone were also in the range of 78 per cent, demonstrating a disappearance of approximately 10 per cent of unsaturated ketonic material.

**Detection of Steroids by Paper Chromatography**—Aliquots of crude extracts (equivalent to 600 to 1200 $\gamma$ of starting steroid) were pipetted onto paper strips 1 cm. wide and developed for various lengths of time. Under these conditions products at a minimal concentration of 5 per cent of the starting material should have been detected. A summary of the findings is presented in Table III. The mobility rates of compounds are expressed in ratio ($R$) to the mobility of cortisone ($C_s$) and adrenosterone ($A_d$) when the chromatograms were developed with toluene and ligroin respectively.

**Blood blanks** gave a faint ultraviolet absorption at $R_{C_s} 0.05$. **Recovery experiments** showed in addition the cortisone zone only. In **blood incubations** a further zone at approximately $R_{C_s} 0.28$ was detected by TPTZ. For the purpose of a quantitative determination of the material in this area, the eluates were rechromatographed in one instance to remove the accompanying yellow color. Using the blue tetrazolium method (17) with tetrahydrocortisone-($3\alpha$,normal) as standard, 9.5 per cent product was found to be present. Since the disappearance of the $\alpha,\beta$-unsaturated ketonic material after the blood incubations amounted to 10 per cent (see Table II), this fraction accounted for the major transformation product (or prod-

**Table II**

<table>
<thead>
<tr>
<th>Recovery of $\alpha,\beta$-Unsaturated Ketonic Material after Incubation and Perfusion</th>
<th>No. of experiments</th>
<th>Per cent recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery experiments</td>
<td>9</td>
<td>88 ± 3</td>
</tr>
<tr>
<td>Blood incubation (normal)</td>
<td>9</td>
<td>78 ± 5</td>
</tr>
<tr>
<td>&quot; (abnormal)†</td>
<td>2</td>
<td>57 ± 3</td>
</tr>
<tr>
<td>Adrenal perfusion</td>
<td>2</td>
<td>62 ± 2</td>
</tr>
</tbody>
</table>

* Expressed in per cent of the starting material.
† Eleven samples of bovine blood were used in this series; three were aseptically drawn and eight collected from the severed neck vessels. The two abnormal instances cited were observed with blood from the latter source.
ucts) of blood action on cortisone. When this material was mixed with tetrahydrocortisone-(3α, normal) and developed on paper, no resolution occurred. Color reactions on the paper were identical with those seen with the pure tetrahydrocortisones 3α, normal and 3β, allo. The area containing this material was thus designated as the "tetrahydro zone."

**Table III**

*Summary of Substances Detected through Analyses by Paper Chromatograms*

<table>
<thead>
<tr>
<th>Designation of zone</th>
<th>Experiment</th>
<th>Ultraviolet absorption</th>
<th>TPTZ</th>
<th>2,4-Dinitrophenylhydrazine</th>
<th>Zimmermann reagent</th>
<th>Fuming sulfuric acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>From crude extracts</strong>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RC</strong></td>
<td>0.05</td>
<td>a, b, c, d, b, c, d</td>
<td>+ (+)</td>
<td>Orange</td>
<td>Blue-gray</td>
<td>Yellow after 1 min., yellow fluorescence†</td>
</tr>
<tr>
<td><strong>RC</strong></td>
<td>0.28</td>
<td>c, d</td>
<td>− + −</td>
<td>Gray</td>
<td>Brown-yellow, yellow fluorescence‡</td>
<td></td>
</tr>
<tr>
<td><strong>RC</strong></td>
<td>0.40</td>
<td>d</td>
<td>+ + −</td>
<td>?</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td><strong>From purified fractions of Perfusion A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RAdr</strong></td>
<td>2.1</td>
<td></td>
<td>− + Yellow</td>
<td>Gray</td>
<td>Purple</td>
<td>Light yellow fluorescence‡</td>
</tr>
<tr>
<td><strong>RAdr</strong></td>
<td>0.69</td>
<td></td>
<td>+ + (+)</td>
<td>−</td>
<td>&quot;</td>
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<tr>
<td><strong>RAdr</strong></td>
<td>0.21</td>
<td></td>
<td>− + −</td>
<td>Orange</td>
<td>Purple</td>
<td>&quot;</td>
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<tr>
<td><strong>RAdr</strong></td>
<td>0.18</td>
<td></td>
<td>− + −</td>
<td>&quot;</td>
<td>&quot;</td>
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</tr>
<tr>
<td><strong>RAdr</strong></td>
<td>0.70</td>
<td></td>
<td>− + −</td>
<td>&quot;</td>
<td>&quot;</td>
<td></td>
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<tr>
<td><strong>RAdr</strong></td>
<td>0.34</td>
<td></td>
<td>− + −</td>
<td>&quot;</td>
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</table>

RC, and RAdr are the ratios of the mobility of the unknown compounds to those of cortisone and adrenosterone, respectively, in the toluene- or ligroin-propylene glycol system.

* Zones were found in the following experiments: (a) blood blanks, (b) recovery experiments, (c) blood incubations, and (d) adrenal perfusions.
† Under ultraviolet light.

The possibility that this zone contained a mixture of substances could not be excluded. Model runs with pure samples of steroids in the toluene-propylene glycol system over a development period of 60 hours gave the following picture: Tetrahydrocortisones 3β, allo and 3α, normal were not resolved. However, the 3β, allo and the 3α, allo Goals derivatives separated clearly, giving rise to a displacement effect; the RC was found to be 0.23

13 This compound was obtained from Mr. E. Caspi.
and 0.43 respectively. Other steroids than those tested will have similar mobility rates and might be contained in the tetrahydro zone.

With the adrenal perfusions these same zones were again evident. The material in the tetrahydro zone of the (corresponding instance) Perfusion A amounted to 8.4 per cent. An additional compound at $R_v 0.40$ was detected by ultraviolet absorption (Table III).

**Table IV**

*Silica Gel Chromatography of Extract from Perfusion A*

3.0 gm. of dried extract dissolved in benzene were placed on 90 gm. of silica gel (1.8 X 52 cm.) in benzene-hexane, 1:1. 200 ml. of solvent were used per fraction.

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Eluates</th>
<th>Weight</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3</td>
<td>Benzene, benzene-ether, 4:1, 2:1</td>
<td>2027</td>
<td>Waxy substance</td>
</tr>
<tr>
<td>4-7</td>
<td>Benzene-ether, 1:1-1:4</td>
<td>71</td>
<td>Brown syrup</td>
</tr>
<tr>
<td>8-12</td>
<td>Ether, ether-ethyl acetate, 6:1</td>
<td>36</td>
<td>Few crystals, m.p.&gt;300°</td>
</tr>
<tr>
<td>13-14</td>
<td>Ether-ethyl acetate, 4:1, 3:1</td>
<td>7</td>
<td>Brown syrup</td>
</tr>
<tr>
<td>15</td>
<td>&quot;</td>
<td>13</td>
<td>Few crystals</td>
</tr>
<tr>
<td>16-18</td>
<td>&quot;</td>
<td>247</td>
<td>M.p. 193-220°</td>
</tr>
<tr>
<td>19-22</td>
<td>&quot;</td>
<td>383</td>
<td>Brown syrup</td>
</tr>
<tr>
<td>23-31</td>
<td>&quot;</td>
<td>70</td>
<td>Amorphous material</td>
</tr>
<tr>
<td>32-38</td>
<td>Chloroform, chloroform-methanol, up to 1:2</td>
<td>43</td>
<td>Dark syrup</td>
</tr>
<tr>
<td>39-40</td>
<td>Methanol</td>
<td>28</td>
<td>&quot;</td>
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<tr>
<td></td>
<td>Total</td>
<td>2925</td>
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</table>

**Isolation of Steroids from Adrenal Perfusates**

*Extract A*—The extract (3.0 gm.) of adrenal Perfusate A was dissolved in benzene and chromatographed on silica gel (Table IV). The eluted solid from Fractions 16 to 18 was crystallized with acetone and yielded 171 mg. of a substance with melting at 209–223°. No depression of the melting point was observed when cortisone was admixed.

Fractions 1 to 6 (2087 mg.) were distributed between hexane and 40 per cent aqueous methanol, yielding a residue of 76 mg. from the methanol layer. No steroids were observed on paper chromatogram analysis. Aliquots of other fractions were also analyzed by this method.

In Fractions 8 to 10 four compounds showing the typical Zimmermann color reaction for 17-ketosteroids were detected (Table III). The substances with $R_{Adr} 1.0$ and 0.18 were present in the highest concentration.
They gave only one spot each when run in a chromatogram mixed with adrenosterone and \( \Delta^1 \)-androstene-11\( \beta \)-ol-3,17-dione respectively. The remainder of these combined fractions (approximately 80 per cent) was chromatographed on a paper strip 17 cm. wide for 24 hours in ligroin. The two zones were eluted individually and rechromatographed on narrower paper strips. From the zone with \( R_A = 1.0 \), 210 mg. of syrup were eluted, which permitted identification of the material (see below). The zone with \( R_A = 0.18 \) yielded 1.4 mg. of syrup, from which 0.7 mg. of crystals previously found (m.p. >330°) separated. Fractions 11 and 12 contained very little of the compound (or compounds) with \( R_A = 0.18 \) only.

A substance with \( R_C = 2.1 \), not detected previously, was found to be present in Fractions 13 and 14 and in a mixture with cortisone in Fraction 15. A mixed chromatogram with normal dihydrocortisone gave a uniform spot. The area containing this material was designated as the “dihydro zone.”

The mother liquors of Fractions 16 to 18 consisted of cortisone only. Fractions 23 to 31 contained the compound (or compounds) with \( R_C = 0.05 \) in small quantities, demonstrating at this time a faint pink color with TPTZ. In Fractions 35 to 37 the material with \( R_C = 0.40 \) was found.

Fractions 19 to 22, which contained a mixture of tetrahydro substances and cortisone, were subjected to an aqueous methanol-hexane distribution and afforded 128 mg. of dark syrup from the methanol layer. This residue was subsequently chromatographed on 9 gm. of silica gel. The separation, however, was unsatisfactory. The fractions containing mixtures of steroids were subjected to paper chromatography. A total of 20 mg. of syrup was eluted from the tetrahydro zone. At this time a trace of another substance with \( R_C = 0.69 \) (Table III) was detected.

**Extract B**—The purified extract (797 mg.) from adrenal Perfusion B was processed specifically for the isolation of tetrahydro products. 165 mg. of cortisone, m.p. 196–214°, were separated by filtration from the extract moistened with chloroform. The mother liquor was placed on nine paper strips 17 cm. wide and developed for 36 hours at 30° with toluene. After drying the paper sheets in an air draft, the locale of the tetrahydro zone was determined with the TPTZ reagent to be 3 to 7 cm. from the starting line. These areas were cut out and eluted with methanol and yielded 42 mg. of yellow syrup. Residual cortisone and other impurities were removed through rechromatography on a single paper strip. The purified eluate weighed 29 mg.

**Presence of Compound with \( R_C = 2.1 \)**—Extracts of 75 ml. of blood aliquots of both perfusions and corresponding blood incubations were placed on individual paper strips 5 cm. wide and developed in the toluene-propylene glycol system until the cortisone areas had moved to 10 ± 3.5 cm. from the
starting lines. The zones below this area (14 to 25 cm.) were cut out and eluted, and the residues rechromatographed on strips 1.5 cm. wide. The compound, previously noted in chromatograms of perfusion Extract A, moving with $R_c$ 2.1, was detected only in the material from the two adrenal perfusions, but not in that of the blood incubations. Under the conditions applied any quantities of this substance exceeding a concentration of 0.5 per cent of the starting material should have been detected.

**Identification of Crystalline Products from Adrenal Perfusions**

*Zone with $R_c$ 2.1 Constituting Alodihydrocortisone—* 90 per cent of Fractions 13 to 14 from the chromatogram of the extract of Perfusate A (see Table IV) was placed on a paper strip 7 cm. wide impregnated with 40 per cent methanolic propylene glycol and developed for 17 hours at 30° in toluene. 90 per cent of Fraction 15 was chromatographed on a sheet 17 cm. wide. The areas with $R_c$ 2.1 (detected by TPTZ) between 10 and 14 cm. from the starting line were cut out and eluted with acetone. A total of 7 mg. of solid was obtained, which after two crystallizations from acetone-pentane, gave 2.5 mg. of fine needles (m.p. 195-205°). These crystals plus a second crop (a total of 4.5 mg. or 1 per cent of the starting material) were acetylated in the usual manner. 5.7 mg. of solid residue were obtained and yielded, after two crystallizations from acetone-ether, 3.3 mg. of needles (m.p. 229-236°). A mixed melting point with the reference sample of alodihydrocortisone did not show a depression. Proof of identity was established by comparison of their infra-red spectra.

*Zone with $R_c$ 0.28 Containing Tetrahydrocortisone-(3β,allo)—* Attempts to obtain crystals directly from the paper eluates of the zone with $R_c$ 0.28 were unsuccessful. The residue was acetylated and after several crystallizations from ethyl acetate a small quantity of needles, melting at 211-221°, were obtained. A mixed melting point with the reference sample of tetrahydrocortisone-(3β,allo)-diacetate did not show a depression. The identity of the substance was proved by infra-red spectroscopy.

The difficulties encountered in the purification of the crude material as well as indication from the infra-red analyses of the mother liquors suggested that these fractions consisted of more than the $3\beta$, allo derivative isolated above. Attempts to resolve the acetylated components with various paper chromatogram systems failed. A sample of the original unacetylated material was chromatographed in the chloroform-formamide system (24) for very slow moving steroids. After 16 hours of development at 22° the substance (or substances) with $R_c$ 0.28 had moved 20.0 cm. and a trace of a substance with a calculated $R_c$ 0.21 was separated (Table III).

*Zone with $R_{Adr}$ 1.0 Containing Adrenosterone—* From the 2.0 mg. of eluted syrup (see above), 0.7 mg. of crystals melting at 220-222° was
obtained. Mixed melting point and infra-red analysis showed that the compound isolated was adrenosterone.

**Formation of Adrenosterone from Cortisone**

Blood Incubation and Subsequent Extraction with Charcoal—250 mg. of cortisone in 12 ml. of propylene glycol were mixed with 1.66 liters of blood and circulated through the perfusion apparatus (in the absence of adrenal glands) for 3 hours at a medium flow rate. The steroids were then adsorbed at 4° three times on 30 gm. of charcoal (Darco G-60) and treated as detailed elsewhere (2, 25). The vacuum-dried charcoal was eluted in a Soxhlet apparatus consecutively with methylene chloride, benzene, and ethyl acetate. The dried extracts obtained were combined (560 mg.) and chromatographed on silica gel. Only 10 mg. of cortisone (m.p. 195–223°) were recovered. This was converted to the acetate, which melted at 236–241.5°. A mixed melting point and infra-red analysis confirmed its identity. Another product (20 mg.), after recrystallization from ether, melted at 222.5–224° and had [α]_D^21 +309° ± 6° (c, 0.716 in chloroform). It did not show a melting point depression when mixed with an authentic sample of adrenosterone. The identity of these substances was substantiated by infra-red analyses.14

C_{19}H_{24}O_{3}. Calculated, C 75.97, H 8.05; found, C 75.74, H 8.03

Cortisone Recovery, Control Experiments—4 mg. of cortisone in 0.8 ml. of propylene glycol and 100 ml. of water were shaken with 6 gm. of Darco at 4° for 40 hours. The dried charcoal was extracted with the series of low boiling solvents mentioned above. An aliquot of the combined extracts showed a 44 per cent recovery of material with the α,β-unsaturated ketonic grouping. Another aliquot was put on paper strips 1 cm. wide impregnated with methanolic propylene glycol (1:1) and developed for 1½ hours in toluene. A substance moving at a rate similar to adrenosterone was detected with the Zimmermann reagent. The intensity of this color was compared with reference strips run in parallel containing 25 and 50 γ of adrenosterone mixed with an appropriate amount of (blank) charcoal extract. Approximately 14 per cent of the starting material was calculated as being transformed into adrenosterone.

In a second experiment in which the extraction of the cortisone adsorbed on charcoal was effected with several portions of acetone at 40° under a nitrogen atmosphere, the recovery was only 25 per cent. An aliquot was distributed between benzene and water and the residue of the benzene frac-
tion chromatographed on paper. Practically no Zimmermann color appeared in the adrenosterone area.

Effect of Ether Peroxides—4 mg. of cortisone dissolved in 5 ml. of chloroform were added to 250 ml. of water-saturated ether containing peroxides in a concentration of approximately 0.13 N. After standing for 48 hours at room temperature, the solution was divided into two equal parts. One part was shaken, for reduction of the peroxides, with 2 ml. portions of 5 per cent ferrous sulfate, until no further color change was evident, and then with six portions of 1.5 ml. of saturated sodium chloride. The other portion was treated with the sodium chloride only. Aliquots of the residue of these extracts were analyzed on paper for the presence of adrenosterone. The sample shaken with ferrous sulfate showed only a trace of this steroid, whereas the non-treated sample yielded a product (estimated at 10 per cent) which moved at the same rate that adrenosterone did. A mixed chromatogram on paper in the ligroin-propylene glycol system (15 hours development) with an authentic sample of adrenosterone gave only one spot.

Effect of Temperature—4 mg. of cortisone in stoppered tubes, one sample in the dry state and the other in 0.4 ml. of propylene glycol, were heated at 95° ± 2° for 24 hours. At the end of this period, the melting point and the extinction at 240 μ of the dry sample were identical with those taken before heating. No adrenosterone was detected on analysis by paper chromatogram. The sample which had been heated in propylene glycol showed a decrease of 5 per cent of the ultraviolet adsorption reading. On paper a product was found which moved at the same rate that adrenosterone did and was estimated as 15 per cent. The identity was substantiated by a mixed chromatogram with an authentic sample of adrenosterone.

DISCUSSION

Recent studies in vitro with rat tissues have shown that an extensive "inactivation" of corticosteroids (desoxycorticosterone, cortisone) occurred not only in liver but also in other tissues. This conclusion was drawn by determination of specific groupings of the molecule of the incubated steroids (26, 27) and through bioassays involving liver glycogen deposition (28, 29). In the case of cortisone the kidney and spleen in addition to the liver have been found to be particularly active. Other experiments carried out by Louchart and Jailer with blood serum demonstrated an inactivation of 12 to 16 per cent of the steroid, which was considered an insignificant change in terms of the glycogenic assay employed (28).

In the present work an 11 per cent reduction of cortisone by whole bovine blood has been demonstrated. The reduced steroids which appeared to be formed are generally considered to be devoid of the recognized cortin
activities (30). Whether these types of reduced derivatives exercise other biological functions is as yet unknown. It would also be of interest to extend incubation studies on a larger scale and with the use of human blood, which is the one abundantly available tissue of this species whose steroid metabolites have been so extensively elucidated in the urine.

The influence of adrenal tissue on cortisone has been examined previously. Schneider and Horstmann (27) noted a small decrease of the steroid in incubations with rat adrenal homogenates. In the perfusions of bovine adrenals described in this paper the disappearance of cortisone amounted to 29 per cent. Approximately 11 per cent could be accounted for as reduced derivatives. The possible sources where the loss of the remaining 18 per cent may have occurred are (a) through adsorption to the tissue of the glands; (b) through conjugation to water-soluble products; (c) through formation of small amounts of steroids not detectable with the color reactions employed; or (d) through degradation of the steroid molecule.

Of the reduced products of cortisone in this blood-adrenal system two substances were isolated and identified: alldihydrocortisone and tetrahydrocortisone-3β,allo or Reichstein’s Substance D. The dihydro compound appeared only in the extracts of blood which had been perfused through adrenal glands. The more polar reduced products were found in both the perfusates and blood incubations. Whether these fractions in the tetrahydro zone consisted of an identical single compound or unresolved mixtures was not ascertained.

Tetrahydrocortisone-3β,allo was first isolated in 1936 from beef adrenal extracts by Reichstein (31). The 3β,allo tetrahydro derivatives of the other five “active” cortical hormones, exclusive of desoxycorticosterone, were also found (32). The corresponding alldihydro derivatives are not known from this source. However, they have been detected in these laboratories on various occasions after adrenal perfusion (e.g. from Δ4-androstene-11β-ol-3,17-dione (33) and Δ4-pregnen-17α-ol-3,11,20-trione (21-desoxycorticisone)16).

The instability of cortical hormones to heat, alkali, aluminum oxide, ether peroxides, etc., has been long recognized. However, the resulting transformations have been elucidated in only a few cases (34). In this communication the degradation of cortisone to adrenosterone by charcoal, heat, and ether peroxides under well defined conditions has been illustrated. This would support the opinion of Reichstein and Shoppee that adrenos-
terone isolated from beef adrenal extracts may be an artifact of the break-
down of C₂₁ steroids (32). Therefore it would be premature to interpret
the small amounts of adrenosterone (and the other "17-ketosteroids"),
detected after adrenal perfusion and direct extraction, as metabolites of
corticosterone. In this connection, the recent demonstration that Δ⁴-andros-
tene-11β-ol-3,17-dione can be formed by adrenal tissue (33, 25, 35) from
C₁₉ precursors should be recalled.

The author wishes to acknowledge his appreciation to Dr. G. Pincus for
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ful assistance throughout the course of this investigation. Thanks are
given to Dr. M. Hayano for help in writing the manuscript.

SUMMARY

1. Ethyl acetate has been found advantageous for the direct extraction
of corticosteroids from blood.
2. Incubation of cortisone and adrenosterone with bovine blood resulted
in a reduction of the α,β-unsaturated ketonic grouping of these steroids.
Essentially all of the reduction product of cortisone was accounted for by
one or more substances which moved on paper chromatograms at a rate
identical with that of the tetrahydrocortisones (3-OH,5-H trans).
3. Adrenal perfusion with cortisone in blood has led to the isolation of
allopregnane-3β,17α,21-triol-11,20-dione and allopregnane-17β,21-diol-3,
11,20-trione. Other transformation products also appeared to be present
in minute quantities.
4. A degradation of cortisone to adrenosterone was shown to occur on
 treatment with charcoal or on exposure to peroxide oxides or elevated tem-
peratures (95°). Cortisone in the solid state was heat-stable.
5. A preparation for adrenosterone from cortisone is described.

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CHEMICAL TRANSFORMATIONS OF STEROIDS BY ADRENAL PERFUSION:
V. BLOOD INCUBATIONS AND PERFUSIONS WITH CORTISONE
André S. Meyer