THE COLORIMETRIC MICRODETERMINATION OF CORTICOIDS BY USE OF 4,7-DIPHENYL-1,10-PHENANTHOLINE

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With the rapid development of information concerning the isolation, identification, and physiological levels of corticosteroids, a relatively simple microchemical method for their quantitative estimation appears to be desirable.

Since the known biologically active adrenal cortical hormones contain a primary \( \alpha \)-ketol function, an \( \alpha,\beta \)-unsaturated 3-ketone group, or both, several methods based upon the reducing capacity of the cortical hormones have been proposed for their quantitative analysis. Thus, in 1945, Talbot et al. (1) used the reduction of cupric ion, in 1946 Heard and Sobel (2) used the reduction of phosphomolybdic acid, and, in 1953, Chen and collaborators (3) applied the use of tetrazolium salts. In general, in addition to other limitations, the prevailing methods lack sensitivity. In view of the relatively small concentrations of corticosteroids in normal human urine, processing of large amounts of urine has been necessary for reducing methods previously described. Since the possible presence of non-alcoholic reducing lipides has been reported in urine extracts (4), it is felt that a very sensitive method for corticosteroids will minimize the discrepancies reported.

Preliminary work in the field of analytical reagents used in oxidation-reduction systems indicated that the heterocyclic nitrogen compounds of the phenanthroline type might be more sensitive and useful reagents, and possibly an improvement over the use of existing reagents for estimating cortical steroids.

The present investigation concerns the development of a sensitive and simple method based on the reduction of ferric chloride by corticosteroids, and the estimation of the ferrous iron by certain heterocyclic nitrogen compounds, to yield a stable red color (ferroin reaction). Of the various phenanthroline derivatives that could be used for reduction study, the reagent 4,7-diphenyl-1,10-phenanthroline prepared by Case (5) was selected as the analytical reagent of choice. This reagent is also known as bathophen-
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anthroline and forms a molecular complex ferrous ion (6) with a wave length of maximal absorption at 533 m\(\mu\) and a molecular extinction coefficient of 22,400.

The ferroin reaction was applied by McFarlane (7) in 1936 in a study of the relative capacities of tissue extracts to reduce ferric iron. He employed dipyridyl to determine the quantity of ferrous iron produced when ferric iron was reduced by the biological material. The same reagent, in an aqueous medium, has recently been used by Sullivan and Clarke (8) in a highly specific procedure for ascorbic acid. The use of heterocyclic nitrogen compounds of the phenanthroline type as possible reagents for estimating reducing corticoids is reported in this paper.

**EXPERIMENTAL**

In preliminary qualitative experiments, the possible use of the reagents \(\alpha,\alpha'-dipyridyl\) and 1,10-phenanthroline was explored. It was observed that, when the solutions of the various corticoids in the presence of ferric chloride and either dipyridyl or 1,10-phenanthroline were brought to approximately pH 5.6 with pyridine as the base and the tubes were warmed by being placed in a heated water bath, a color change from straw-yellow to red gradually resulted.

The tubes containing the following cortical hormones gave the red color: deoxycorticosterone, 17-hydroxydeoxycorticosterone, corticosterone, 17-hydroxy-11-dehydrocorticosterone, 17-hydroxyxycorticosterone, and aldosterone. Under similar conditions, but without the addition of corticoid, no color change resulted. Corticosteroids with the primary alcoholic group acetylated did not yield the red color. When steroids such as testosterone and progesterone were tested under the conditions mentioned above, no color change was produced. Since the \(\alpha,\beta\)-unsaturated 3-ketone group is common in testosterone, progesterone, and active corticosteroids, and since progesterone, testosterone, and the esters of the corticoids did not reduce the ferric chloride, it would indicate that the reduction of ferric chloride is dependent upon the free primary \(\alpha\)-ketol group (\(-C-\text{CH}_2\text{OH}\)) in the side chain attached to C-17 of the corticoids. Testosterone and progesterone reduce the phosphomolybdate reagent (2), and also reduce to some extent the tetrazolium salts (3), but they do not reduce the cupric ion in the method of Talbot et al. (1).

Since the sensitivity of the 1,10-phenanthroline was found to be less than that of the \(\alpha,\alpha'-dipyridyl\) in a tentatively adopted procedure, it was not investigated extensively. Studies were undertaken to investigate the optimal conditions for color development with dipyridyl in the presence of
reducing corticoids. Evidence indicated that the type of organic base used for regulation of the pH, the type of solvent, the concentration of reagents, and the temperature and length of time of heating influenced the final intensity of color. Of the various bases tested, pyridine proved to be the most satisfactory. Maximal reduction resulted at the pH range 5.2 to 5.8. Sensitivity was greatly decreased below pH 3.2 and above pH 6. It will be noted in Table I that bases other than pyridine and formamide used to obtain the required pH conditions were unsatisfactory because of the formation of a turbidity, owing probably to ferric hydroxide. The use of an acetate buffer to produce the required pH conditions was also unsatisfactory because of the formation of a precipitate in the final solution. When formamide was used to obtain pH range 5.2 to 5.8, a precipitate was not produced in the final solution. However, the sensitivity of the pro-

<table>
<thead>
<tr>
<th>Base</th>
<th>pH* of final solution</th>
<th>Reduction of ferric chloride</th>
<th>Turbidity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH</td>
<td>5.6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KOH (alcoholic)</td>
<td>5.6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Benzyl trimethylammonium hydroxide</td>
<td>5.6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Choline</td>
<td>5.6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Formamide</td>
<td>5.2-5.8</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Pyridine</td>
<td>5.2-5.8</td>
<td>+++</td>
<td>-</td>
</tr>
</tbody>
</table>

* Measured with Beckman pH meter, model G.

Table I

Bases Tested with α,α'-Dipyridyl As Reagent

The solvent of choice for the corticosteroids was found to be ethyl alcohol (95 per cent). Just as in the case of pyridine, ethyl alcohol required purification before use to remove interfering oxidizing or reducing agents present. Alcohols other than ethanol were found unsuitable even after purification. The ethyl alcohol was treated as in the purification of pyridine; that is, it was refluxed over potassium hydroxide (1 gm. of KOH per 100 ml.) for approximately 30 minutes in an all-glass apparatus. After the period of refluxing, the respective solutions (alcohol and pyridine) were distilled, and the first and last fractions were discarded. The selected fractions were tested for the presence of reducing substances by running a few ml. of each of the collected solutions according to the analytical procedure with dipyridyl or Bathophenanthroline. At the end of the 45 minute heating period of the analytical procedure, the solutions were straw-yellow in color; a red or orange-red color indicates that reducing
substances are still present. All samples of ethyl alcohol issued by the pharmacy of this hospital and all samples of reagent grade pyridine were found suitable after a single purification.

The most satisfactory conditions for the temperature of the water bath and the period of heating were found to be as follows: a constant temperature water bath set at 59-60° and a heating period of 45 minutes. Under these conditions, a series of determinations on solutions of hydrocortisone, in concentrations varying from 0 to 100 γ, revealed that the intensity of the color varied directly with the quantity of reducing substance present. Agreement with Beer's law was also established for cortisone and deoxycorticosterone. Standard curves for any one of the corticoids tested in the

**Table II**  
Optical Density* of Bathophenanthroline-Ferrous Complex  
Produced by 10 γ of Steroids

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Structure</th>
<th>Optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxycorticosterone</td>
<td>(\Delta^4)-Pregnen-21-ol-3,20-dione</td>
<td>0.24</td>
</tr>
<tr>
<td>&quot; acetate</td>
<td>&quot; acetate</td>
<td>0.002</td>
</tr>
<tr>
<td>Compound E (Kendall)</td>
<td>(\Delta^4)-Pregnen-17β,21-diol-3,11,20-trione</td>
<td>0.22</td>
</tr>
<tr>
<td>&quot; acetate</td>
<td>&quot; acetate</td>
<td>0.002</td>
</tr>
<tr>
<td>&quot; F (Kendall)</td>
<td>(\Delta^4)-Pregnen-11,17β,21-triol-3,20-dione</td>
<td>0.20</td>
</tr>
<tr>
<td>&quot; B &quot;</td>
<td>(\Delta^4)-Pregnen-11,21-diol-3,20-dione</td>
<td>0.21</td>
</tr>
<tr>
<td>Progesterone</td>
<td>(\Delta^4)-Pregnen-3,20-dione</td>
<td>0.002</td>
</tr>
<tr>
<td>Testosterone</td>
<td>(\Delta^4)-Androstene-17α-ol-3-one</td>
<td>0.002</td>
</tr>
</tbody>
</table>

* Beckman DU spectrophotometer, Corex cell 1 cm., 533 mμ, slit 0.11.

procedure may thus be applied to estimations of any reducing steroid in terms of the reducing equivalent of whichever pure compound is selected as the standard reference.

The steroids used in this study were obtained through the courtesy of several pharmaceutical laboratories and research organizations. Equal concentrations of two samples of 17-hydroxy-11-dehydrocorticosterone (cortisone) obtained from two different sources gave the same results in terms of optical density.

Table II shows the optical density readings obtained with 10 γ of a number of steroids with bathophenanthroline as the reagent. It is evident that acetylation of the primary alcohol group of the corticoids ties up the reducing group and that in the new procedure presumably no hydrolysis of the acetate results to release the free ketol. It is obvious, therefore, that the esters of the corticoids may not be used as standards in the new procedure.

To determine whether the daylight and the fluorescent lighting in the
laboratory had any effect upon solutions of corticoid in the presence of ferric chloride, the colorimetric procedure was run in test tubes, the outer surfaces of which were coated with black enamel paint. However, whether the tubes were coated or non-coated, there was no indication of photosensitive changes in the solutions.

Two procedures for estimating corticosteroids are presented in this paper (see Figs. 1 and 2). The first procedure describes the use of the reagent dipyridyl and is used when estimating reducing steroids in amounts greater than 25 μg. The second procedure describes the use of the analytical reagent, 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline), and, when maximal sensitivity is necessary, should be used when measuring small amounts, i.e. several micrograms of reducing steroids.

Procedure A. α,α'-Dipyridyl Procedure

Reagents—
Ethanol. U. S. P. ethyl alcohol refluxed over potassium hydroxide (1 gm. per 100 ml.) for 30 minutes and distilled. The middle fraction collected.
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Pyridine. Reagent grade refluxed over potassium hydroxide (1 gm. per 100 ml.) for 30 minutes and distilled. The fraction of boiling point 114–115° collected.

α,α′-Dipyridyl. 1.2 per cent solution in purified ethanol is stored in a glass-stoppered bottle and should be colorless.

Ferric chloride (hydrated). Reagent grade should be pulverized if necessary. An 80 mg. portion is dissolved in 20 ml. of ethanol in an amber glass-stoppered bottle and is prepared fresh each day.

Standard stock solution. 25 mg. of any one of the pure reducing corticoids dissolved in 25 ml. of ethanol are stored in the refrigerator in a glass-stoppered bottle.

Working standard. The stock standard is diluted 1:10. Concentrations of 25, 50, 75, and 100 γ per ml. in purified ethanol are used to prepare the standard reference curve.

Colorimetric “Procedure A.” Test tubes approximately 1.5 cm. X 15 cm. are used. To 2 ml. of ethanolic solution of the steroid are added 0.5 ml. of alcoholic dipyridyl solution and 0.5 ml. of alcoholic ferric chloride solution. The tube is shaken for 15 to 20 seconds. Then 1 ml. of pyridine is added and the tube is again shaken for 15 to 20 seconds. The tube is placed in a water bath set at 59–60° for 45 minutes. (The level of the solution in the tube should be approximately at the level of the water of the bath.) The tube is then cooled for a few minutes by immersing it in a beaker of cold water. The solution is read at 520 μ. (For the blank determination, purified ethanol is run simultaneously.)

Procedure B. 4,7-Diphenyl-1,10-Phenanthroline Procedure

Reagents—
Ethanol. U. S. P. ethyl alcohol refluxed over potassium hydroxide (1 gm. per 100 ml.) for 30 minutes and distilled. The middle fraction collected.

Pyridine. Reagent grade refluxed over potassium hydroxide (1 gm. per 100 ml.) for 30 minutes and distilled. The fraction of boiling point 114–115° collected.

Bathophenanthroline (G. Frederick Smith Chemical Company, Columbus, Ohio). A 200 mg. portion is dissolved in 50 ml. of ethanol. (Warm the solution slightly to dissolve the reagent completely.) The solution should be colorless and stored in a glass-stoppered bottle.

Ferric chloride (hydrated). Reagent grade should be pulverized if necessary. A 20 mg. portion is dissolved in 20 ml. of ethanol in an amber glass-stoppered bottle and is prepared fresh each day.

Working standards. The stock standard described under “Procedure
A" is diluted 1:50. Concentrations of 2 to 15 \( \gamma \) per ml. in ethanol are used for the standard curve.

Colorimetric "Procedure B." As described under "Procedure A," with 0.5 ml. of bathophenanthroline reagent in place of the dipyridyl reagent. Readings are made at 533 m\( \mu \). (For the blank determination, purified ethanol is run simultaneously.)

**SUMMARY**

1. Two simple colorimetric procedures have been presented for the estimation of small amounts of corticosteroids by the use of certain heterocyclic nitrogen compounds as reagents. The first procedure describes the use of the reagent \( \alpha, \alpha' \)-dipyridyl and is used when estimating reducing steroids in amounts greater than 25 \( \gamma \). The second procedure describes the use of the reagent bathophenanthroline and is used in microdeterminations when maximal sensitivity is necessary.

2. Acetylation of the primary alcohol function of the corticoids prevents reduction, and consequently the esters of the various corticoids cannot be used as standards and cannot be tested with the new procedure.

3. The colorimetric reactions described are given by steroids possessing the free \( \alpha \)-ketol grouping \( \text{--C--CH}_2\text{OH} \) at the C-20 and C-21 positions. Steroids possessing the \( \alpha, \beta \)-unsaturated 3-ketone group, but not the C-17-substituted \( \alpha \)-ketol group in the side chain such as testosterone and progesterone, yielded no color when subjected to these colorimetric procedures.

The colorimetric procedure is being applied to the estimation of the neutral lipid-soluble reducing substances of urine in normal and pathological conditions and under corticotropin stimulation.

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**BIBLIOGRAPHY**


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