A COLORIMETRIC REACTION FOR TESTOSTERONE*

BY VIRGIL L. KOENIG, FRANCISCA MELZER, CLARA M. SZEGO, AND LEO T. SAMUELS

(From the Division of Physiological Chemistry, Department of Physiology, School of Medicine, University of Minnesota, Minneapolis)

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Since the discovery of the sex hormones, there has been a great need for suitable rapid chemical methods for the quantitative estimation of these hormones in biological materials. Kober (4) in 1931 was the first to apply a color reaction to the quantitative determination of estrogens. By this technique Kober found that estrogens produced a red color when treated with phenolsulfonic acid and sulfuric acid. Later he (5) found β-naphthol in concentrated sulfuric acid to be superior to the original phenol reagent. Other workers (1-3) have studied the reaction as applied to estrogens and have made various modifications.

It was while studying the Kober reaction that Szego and Samuels (6) discovered the value of guaiacolsulfonic acid as a reagent for estrone. Their technique was essentially that of Kober; namely, the development of color with concentrated sulfuric acid and a solution of potassium guaiacolsulfonate, commercially known as thiocol. When testosterone was tested with this thiocol reagent, a blue fluorescence was given. When copper or iron salts were present, a bright green color developed. The production of this bright green color by the action of thiocol reagent on testosterone offered a possible basis for a quantitative method specific for testosterone. Heretofore, the Zimmermann (7) reaction has been the only colorimetric method for androgens, and

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Reaction for Testosterone

it is not specific, since it is primarily a ketone reagent. Since the thiocol reagent seemed specific for testosterone, it was decided to study the reaction further.

EXPERIMENTAL

The specific directions for carrying out the reaction are as follows: A 10 ml. graduated test-tube containing the testosterone (either dry or in about 0.4 ml. of 95 per cent alcohol) is placed in an ice bath. 2 ml. of concentrated sulfuric acid are added and mixed with a footed stirring rod, care being taken to avoid spattering. The tube is heated for 2 minutes in boiling water without stirring, and then placed in an ice water bath. After 5 minutes, 2 ml. of saturated aqueous thiocol and 0.3 ml. of 1 per cent aqueous copper sulfate are added with stirring. The tubes are reheated in boiling water for 2 minutes. During this period they are stirred three times. The tubes are again placed in the ice water bath and diluted to the 10 ml. mark with 50 per cent sulfuric acid. After being transferred to a colorimeter tube, the solution is read against a blank containing the reagents, but not the hormone, in an Evelyn colorimeter equipped with a 635 mμ filter.

The production of the green compound apparently involves an oxidation. Some green color is produced if the solution without copper or iron salts is vigorously stirred in the boiling water bath. Copper sulfate is used to accelerate this reaction, because it produces the most consistent results. When ferric chloride is used, the green color tends to fade when a small excess is added. A relatively large excess of copper sulfate can be added without any fading. Stronger oxidizing agents such as hydrogen peroxide and potassium permanganate destroy the green color.

Better results are obtained when the thiocol is recrystallized from 60 per cent alcohol. Since thiocol is known to consist of two isomers, recrystallization further concentrates the predominating isomer, which is presumably the one that is responsible for the production of the color.

The calibration curve in Fig. 1 shows the amount of light that is absorbed by various quantities of testosterone with the 635 mμ filter. Each point on the curve represents the average of many readings. Those points which are off the curve indicate the degree of variation of the readings from the mean. For the smaller
amounts of testosterone the values vary only one or two points from the mean. For 40 $\gamma$ of testosterone the greatest variation from the mean is three or four points. The majority of the values of course fell on or very close to the curve. It seems that from 40 to 80 $\gamma$ the curve veers from Beer's law, and the extent of

![Calibration curve](image)

**Fig. 1.** Calibration curve

**Table I**

<table>
<thead>
<tr>
<th>Testosterone</th>
<th>Colorimetric readings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Determination 1</td>
</tr>
<tr>
<td>$7$</td>
<td>77</td>
</tr>
<tr>
<td>10</td>
<td>79</td>
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<tr>
<td>20</td>
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<td>38</td>
<td>37</td>
</tr>
<tr>
<td>40</td>
<td>27</td>
</tr>
</tbody>
</table>

variation from the mean is about the same as for 40 $\gamma$. Table I shows the colorimeter readings taken for several simultaneous determinations of various amounts of testosterone during 1 day. During a single series of determinations the readings seem to be quite consistent and remain quite consistent throughout the day;
however, from day to day the readings tend to vary more or less. As was indicated in Fig. 1, the variation is never more than three to four divisions on the colorimeter when the reaction is run with 40 $\gamma$ of testosterone and is proportionate to the amount present. For this reason it seems best to run a standard of this strength along with the unknown. For accurate work the amounts in the unknown should then be determined from a curve based on the reading of the simultaneous standard. The curve of Fig. 1 was drawn from the average of a large number of values taken over a period of weeks.

Out of fifteen compounds related to testosterone tested, only three compounds give a green color: testosterone oxime, tes-

tosterone propionate, and androstenedione. The first two are probably hydrolyzed in the reaction medium.

Absorption spectral data indicate that the color produced by androstenedione is identical with that produced by equimolar concentrations of testosterone. The absorption curves for both substances are the same. Fig. 2 shows the absorption curve given by the testosterone reaction. The maximum absorption is at about 6390 Å. This absorption curve was obtained by plotting $D$ versus wave-length. $D$ is equal to $-\log T$. $T$, or transmission, is the ratio, $I/I_0$, where $I_0$ is the incident intensity or the intensity of light passing through the blank, and $I$ is the transmitted intensity or the intensity of light passing through the colored solution. The values of $I$ and $I_0$ were determined by means of a

![Absorption spectral curve of the colored compound](image)
photoelectric spectrophotometer. The colored compound is insoluble in organic solvents except acetone.

The following compounds have been tested and have been found to give negative reactions by the above procedure: ethynyltestosterone, androsterone, dehydroandrosterone, Δ⁵-androstenediol-3-trans-17-cis, androstenedione-3,17, 3,11,17-androstane-trione, Δ⁴-3,11,17-androstenetrione, 11-dehydro-17-hydroxycorticosterone, pregnenin-17-diol-3,17, etioallocholanol-3(β)-17-one, progesterone, cholesterol, estradiol, estrone, and estriol. By inspecting the formulas of the compounds that do give the test, a very close relationship of the compounds to testosterone can be seen.

When testosterone was determined in mixtures of androsterone and dehydroandrosterone, neither androsterone nor dehydroandrosterone interfered appreciably with the production of the testosterone color. Table II gives the results obtained when the mixtures were tested.
Reaction for Testosterone

A study of the recovery of testosterone from tissues and tissue extracts is in progress.

The thiocol was kindly furnished by Hoffmann-La Roche, Inc. The authors are indebted to Dr. H. L. Mason, Dr. R. I. Dorfman, Dr. F. C. Koch, and Dr. R. D. Shaner for supplying various sterols for testing. In obtaining the absorption spectral curves for testosterone and androstenedione the authors are indebted to Dr. E. S. Miller for the use of the photoelectric spectrophotometer.

**Table II**

<table>
<thead>
<tr>
<th>40 γ testosterone with</th>
<th>Transmission per cent</th>
<th>Testosterone found γ</th>
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<tbody>
<tr>
<td>10 γ dehydroandrosterone</td>
<td>27</td>
<td>40</td>
</tr>
<tr>
<td>20 “ “</td>
<td>26</td>
<td>42</td>
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<tr>
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<tr>
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<td>23</td>
<td>48</td>
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<tr>
<td>10 “ androsterone</td>
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<tr>
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<tr>
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<td>26</td>
<td>42</td>
</tr>
<tr>
<td>40 “ “ + 10 γ dehydroandrosterone</td>
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<tr>
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</table>

**SUMMARY**

A color reaction has been developed for testosterone. Of the eighteen compounds besides testosterone tested, only Δ4-androstenedione-3,17, testosterone propionate, and testosterone oxime give the reaction.

From absorption spectral studies of the colored compound, it has been found that its maximum absorption is at about 6390 Å.

Androsterone and dehydroandrosterone do not interfere appreciably with the testosterone color when mixtures are studied.
BIBLIOGRAPHY

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