THE COLORIMETRIC DETERMINATION OF URINARY ESTRIN

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Kober (1931) was the first to suggest a colorimetric test for urinary estrin. He compared the degree of red color developed after treatment with phenolsulfonic acid in a test sample with a standard cresol red solution. Cohen and Marrian (1934) modified the Kober procedure and determined the hormone content of estrone and estriol fractions by comparison with a standard curve from a set of determinations with pure hormone, using a Lovibond tintometer for color standards. Cartland, Meyer, Miller, and Rutz (1935) have also employed the phenolsulfonic acid reagent, using both the Lovibond tintometer and a colorimeter for color comparisons of their extracts against a crystalline hormone standard. These methods have been employed for the quantitative determination of the estrins. Sala (1935) has employed the greenish fluorescence that develops after the hormones are treated with concentrated H₂SO₄ as a qualitative test for pregnancy in the mare. Chevallier, Cornil, and Verdollin (1935) have used the characteristic ultraviolet absorption at 2800 Å to detect estrogenic substances in human pregnancy urine extracted by alkali hydrolysis. Zimmerman (1935) has suggested that the reaction of estrone with m-dinitrobenzene in alkaline alcoholic solution should afford a good test. The violet color that develops occurs also with a number of other ketones. Schmulovitz and Wylie (1935) have used the orange color developed when the estrins are coupled with diazotized p-nitroaniline as a means of detecting these substances in human pregnancy urine, but no separation of estrone and estriol is made in their extractions. David (1934) has noted a typical blue color when estriol crystals are treated with sulfuric acid followed by arsenic acid.
We have inquired into the usefulness and limitations of certain of these procedures for the determination of urinary estrin in human and rabbit urines. Initially we determined the absorption spectrum of the colored compounds formed with crystalline estrone and estriol, using the recording spectrophotometer of Hardy (1935). Our urine extracts were divided into estrone and estriol fractions after the method of Cohen and Marrian (1934). The presumable hormone content of these fractions was determined by comparing the absorption at the color maximum (as shown by the absorption curve) by using appropriate filters of the S series in a Pulfrich photometer. For reasons which will be given later the urine samples were in every set of determinations compared with a set of crystalline hormone standards. The colorimetric assays were compared with bioassays in a number of instances.

**Phenolsulfonic Acid Test**—Since the exact composition of Kober's (1931) reagent is not clear, we have followed, with modifications, the methods of Cohen and Marrian (1934) and Cartland, Meyer, Miller, and Rutz (1935). Phenolsulfonic acid is made up by heating equal weights of phenol crystals and concentrated sulfuric acid at 110–120° for 15 minutes, cooling, and diluting with 2 volumes of sulfuric acid. The hormone or extracts are evaporated to dryness from an alcoholic solution under reduced pressure in a water bath at 50°.

**Cohen and Marrian Test**—According to Cohen and Marrian's procedure the residue is taken up in 2 cc. of the phenolsulfonic acid reagent, heated for 10 minutes in a boiling water bath, and then placed in ice water for about 1 minute. (The solution may be kept in ice at this stage for as long as 2 weeks without any material error occurring.) The solution is then made up to exactly 4 cc. with 5 per cent sulfuric acid, thoroughly mixed, cooled in ice water for about a minute, and transferred into the absorption cell. 10 minutes after mixing, the maximum color intensity develops and a reading is taken in the photometer. The maximum intensity remains constant for some 10 minutes after it is initially attained.

The absorption spectra of the colored compounds developed from crystalline estrone and estriol by this method are given in Fig. 1. It will be noted that the curves for the two substances are remarkably similar, showing two peaks, one at 510 to 512 μ.
and another at 464 $m\mu$. The estriol shows a narrower, sharper band at the first peak and its absorption curve falls more sharply after the second peak. The curve for estradiol given in Fig. 1 shows a broader absorption band and a slight rise into the ultraviolet.\(^1\) We have included the estradiol because Doisy, MacCorquodale, and Thayer (1935) find that it is probably the estrogenic ovarian follicular product.

The standard curves showing the relation between hormone concentration and color absorption were obtained by studying the transmission through an S-50 filter on the Pulfrich photometer. Sample curves presented in Fig. 2 demonstrate that, within the concentration limits employed, the optical densities are directly proportional to the hormone concentration. The zero concentration is obtained from the reagents without added hormone, and the fact that it has a positive value indicates merely that the test reagent has itself a certain amount of color. We have been unable

\[\text{Fig. 1. Absorption spectra of the colored compounds formed with phenolsulfonic acid (Cohen and Marrian's procedure). Upper curve, estrone 33.3 micrograms; middle curve, estriol 32.3 micrograms; lower curve, estradiol 36.54 micrograms. The ordinate shows the extinction (log (I_0/I)); the abscissa, wave-length in } m\mu.\]

\(^1\) The estradiol curve may differ from those of estrone and estriol, either because of slight impurities present or because the crystalline compound used is in fact a mixture of two stereoisomers.
to prepare a standard curve to which we can refer day in and day out. With any given batch of phenolsulfonic acid the curves check fairly well over a limited period of time, but fresh reagent, even when made from materials from the same source, gives a curve which may differ in slope and intercept from all others (see Fig. 2). Accordingly, in making determinations of hormone content in urine extracts we have derived our urine values from a hormone concentration curve made at the same time.

The concentration values for our urine extracts were made upon at least two dilutions of the extracted material. Ordinarily the values so obtained check each other to within 5 to 10 per cent, but occasionally a difference of as much as 50 per cent is observed. In such cases several further determinations are made. Our
hormone extracts are kept in alcoholic solution in the cold; we have observed no change in the colorimetric titer after 8 months.

We have made some effort to check the delicacy of this test by making up extremely dilute solutions of both crystalline hormone and urine extracts. By making ten determinations against an equal number of blanks, it is possible to detect satisfactorily the presence of as little as 0.2 microgram of estrone or estriol. The standard errors of such sets of observations will vary from test to test, however, so that no known number of determinations can be set in advance as the necessary number.

In testing urine extracts the chief obstacle to a complete colorimetric titration is the occasional sample which develops a cloudy suspension after the addition of the 5 per cent sulfuric acid. Fortunately this occurs rarely, and in a second set of determinations the cloudiness may not appear. None the less, certain extracts will repeatedly give this cloudiness, and absorption measurements on such a suspension are of course useless.

The data giving hormone content in the two fractions of various human and rabbit urines are given in Table I. It is immediately obvious that the two rabbit urines tested show a very high titer by color test but no discoverable activity by bioassay. The reason for this difference is made clear when the absorption spectrum of rabbit urine extract is examined. Typical absorption spectra given in Fig. 3 show a fairly regular rise of absorption from the red to the violet. The sharp bands typical of the crystalline hormones are not present, although there is some indication of a slight rise in absorption in the region of the 510 and 460 m\(\mu\) maxima. Inactive materials present in the extracts are responsible for the apparent hormone content.

In human pregnancy urines there are also inactive materials which give a color change in this test. This is particularly evident in the urine of earliest pregnancy, when the color test gives an estimate of about 4 times as much activity as was actually present in the estrone fractions (Urine 3). This overestimation is less marked in the estriol fractions which in later pregnancy (Urides 8 to 11) give in fact a little more activity than expected on the basis of 1.5 rat units per microgram in the color test. If there were in these bioassays 1.8 rat units per microgram of estriol, the color test would agree well with the bioassay of urines taken at 5\(\frac{1}{2}\) months of pregnancy and later.
| Urine No. | Case No. | Stage of cycle | Estrone* | Estriol* | Overestimate
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Color test</td>
<td>Bioassay</td>
<td>Color test</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>Estrus, rabbit</td>
<td></td>
<td></td>
<td>18.6 micrograms per cc. or 55.8 r.u.</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>Rabbit 27-30 days pregnant</td>
<td>150.4 micrograms per cc. or 451.2 r.u.</td>
<td>Negative at 2.7 r.u. per cc.</td>
<td>140.6 micrograms per cc. or 210.9 r.u.</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>19th day of menstrual cycle, in which conception occurred</td>
<td>202 micrograms or 606 r.u.</td>
<td>148</td>
<td>431 micrograms or 947 r.u.</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>5th day 1st mo. of pregnancy</td>
<td>144 micrograms or 432 r.u.</td>
<td>252</td>
<td>604 micrograms or 1812 r.u.</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>4 mos. pregnant</td>
<td>380 micrograms or 1140 r.u.</td>
<td>675</td>
<td>Cloudy</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>4½ &quot; &quot; &quot;</td>
<td>472.6 micrograms or 1317.8 r.u.</td>
<td>450</td>
<td>827.5 micrograms or 1241 r.u.</td>
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<tr>
<td>7</td>
<td>5</td>
<td>5 &quot; &quot; &quot;</td>
<td>221 micrograms or 663 r.u.</td>
<td>380</td>
<td>605 micrograms or 997.5 r.u.</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>5½ &quot; &quot; &quot;</td>
<td>Cloudy</td>
<td>670</td>
<td>975 micrograms or 1462.5 r.u.</td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>6 &quot; &quot; &quot;</td>
<td>348 micrograms or 1044 r.u.</td>
<td>550</td>
<td>1192 micrograms or 1788 r.u.</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>7 &quot; &quot; &quot;</td>
<td>253 micrograms or 759 r.u.</td>
<td>Not assayed</td>
<td>1001 micrograms or 1501.5 r.u.</td>
</tr>
<tr>
<td>11</td>
<td>7</td>
<td>7-9 &quot; &quot; &quot;</td>
<td>297 micrograms or 891 r.u.</td>
<td>670</td>
<td>3600 micrograms or 5800 r.u.</td>
</tr>
</tbody>
</table>

* We assume 1 microgram of estrone = 3 rat units and 1 microgram of estriol = 1.5 rat units.
† Urines 3 to 11 are human urines. ‡ Underestimate.
The complete absorption spectra of the urine fractions indicate that the color due to the inactive substances is represented by materials showing increasing absorption into the violet. This is illustrated in Figs. 4 and 5 where the curve for the earliest pregnancy urine shows the typical bands least sharply and the sharpest

![Graph](image-url)
rise into the violet. The estrone and estriol curves for the 7 to 9 month urines compare very favorably with the curves for the crystalline hormones (Fig. 1), showing rather sharp drops on either side of the maxima and at most only a slight rise in the violet. These urines (see Table I) gave color tests agreeing most closely with the bioassays.

Marrian and Cohen (1935) have stated that their color test is inapplicable to non-pregnancy urines. It is obvious from these data that, in using absorption methods, fairly reliable estimates of the estriol content can be had with urines of 5½ months pregnancy on, but not with earlier pregnancy urines. The estrone content will apparently be overestimated, even with the urines of later pregnancy. The content of active material can be calculated from the absorption spectra of the urine extracts by assuming that

<table>
<thead>
<tr>
<th>Urine No.</th>
<th>Estrone</th>
<th>Estriol</th>
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<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Calculated</td>
</tr>
<tr>
<td>3</td>
<td>148</td>
<td>178</td>
</tr>
<tr>
<td>6</td>
<td>450</td>
<td>495</td>
</tr>
<tr>
<td>11</td>
<td>670</td>
<td>580</td>
</tr>
</tbody>
</table>

the absorption by interfering substances shows a gradual increase into the violet. Since the rise in absorption by the crystalline hormones first becomes marked at 580 mμ, one can make a rough interpolation on the basis of the slope of the curves of the urine extracts over the range 600 to 580 mμ and the rising portion of the curves in the violet. By subtraction at 500 mμ (where the S-50 filter transmits chiefly) the proportion due to inactive material can be estimated and a correction made. By this procedure we have estimated (Table II) the presumable active material in the three urines for which we have complete absorption spectra (Urines 3, 6, and 11). These are at best crude estimates, since we do not know the exact shape of the absorption curves of the inactive materials, and these may, in fact, differ from specimen to specimen. None the less, by this procedure the bioassays and color assays are brought into somewhat better agreement.
tically this procedure can be duplicated with the Pulfrich photometer by taking readings with the S-50 filter, the S-57, S-61, and the S-43 and interpolating on the rough absorption curve so obtained.

**Test of Cartland, Meyer, Miller, and Rutz**—In this test the phenolsulfonic acid reagent is made by heating equal weights of phenol crystals and concentrated sulfuric acid at 110–120° for 15 minutes, cooling, and diluting with 2 volumes of sulfuric acid. The hormone is heated with 0.2 cc. of the reagent for 2 minutes in a boiling water bath, and the mixture cooled under running water for 1 minute; 0.2 cc. of H₂O is added and heated at 125° in a glycerol bath for 2 minutes, the mixture again cooled for 1 minute, and 0.6 cc. of water added. The absorption spectra of the hormones so treated are given in Fig. 6. It will be noted that there is only a single maximum at 514 mλ and that estradiol does not show this maximum clearly. The color fades quite rapidly in this test. This makes its use with impure extracts rather unreliable, since such extracts develop yellowish discolorations, particularly in the estriol fractions, which do not disappear until some time after the typical red color starts to fade. Furthermore the tendency for cloudiness to develop in the extracts is much increased, so that we have few reliable colorimetric determinations of our extracts by this method.

![Fig. 6](image1.png)

**Fig. 6.** Absorption spectra of colored compounds formed with phenolsulfonic acid (procedure of Cartland, Meyer, Miller, and Rutz). The solid line represents estriol, 48.4; the dotted line, estrone, 48.0; the dash line estradiol, 24.0 micrograms. The ordinate shows extinction (log (I₀/I)); the abscissa, wave-length in mλ.

![Fig. 7](image2.png)

**Fig. 7.** Absorption spectra of colored compounds formed after benzoyl chloride treatment. The dash line represents estrone, 35.23; the solid line estradiol, 36.54; the dotted line estriol, 34.08 micrograms. The ordinate shows extinction (log (I₀/I)); the abscissa, wave-length in mλ.
Benzoyl Chloride Test—This test represents a modification of the procedure used by Görtz (1934) for the detection of cholesterol. The solvent is removed from the hormone which is then dissolved in 2 cc. of chloroform. 1 cc. of a 40 per cent solution of zinc chloride in glacial acetic acid is added, followed by the addition of 1.5 cc. of benzoyl chloride, and the mixture is placed in a water bath at 70°. The mixture is allowed to cool with the water bath for 20 minutes, removed, and made up to 5 cc. with chloroform,

![Graph](image)

Fig. 8. A typical concentration curve for crystalline estrone in the benzoyl chloride test. The ordinate shows absorption with the S-50 filter; the abscissa, total concentration of hormone in micrograms.

and the color absorption measured with the S-50 filter on a Pulfrich photometer.

The absorption spectra of the colored compounds obtained with the hormones are given in Fig. 7. The typical absorption curve of the estrone compound is reproduced (except in the violet) by estradiol, but the estrone compound shows a greater rise at the maximum (500 to 504 m\(\mu\)). Estriol, on the other hand, does not give the typical estrone color with benzoyl chloride.
This test should therefore be useful in determining the estrone content of extracts, provided there are no interfering substances causing the same color. It is just as sensitive as the phenolsulfonic acid test and has the advantage that cloudy solutions are not obtained. It suffers from the disadvantage that the reagents appear to change somewhat from day to day (benzoyl chloride is markedly hygroscopic), so that a concentration curve with pure hormone must be prepared for each set of determinations. A typical calibration curve is given in Fig. 8.

In Table III are presented comparative colorimetric assays of estrone fractions of certain human and rabbit urines. The benzoyl chloride determinations are practically identical with the phenolsulfonic acid data for human urines, but with rabbit urines benzoyl chloride tests invariably give a higher titer. The benzoyl chloride determinations with rabbit extracts are approximately twice the phenolsulfonic acid determinations, regardless of the stage of cycle of the rabbit. This implies that the rabbit urine extracts contain certain materials not present in human urines, and that these additional materials are present in constant ratio to the materials revealed by the phenolsulfonic acid test. Alternatively we may infer that there are no additional materials revealed by the benzoyl chloride test, but that the color intensity developed with benzoyl chloride is twice that of the corresponding
intensities of the hormones. This may be true, since we employ
the hormones merely as our color standards for the rabbit urines
which definitely lack appreciable amounts of hormone.

David Test for Estriol—The crystalline hormone is treated with
0.1 cc. of concentrated sulfuric acid, heated for 1 to 2 minutes in
a boiling water bath, cooled, and while cooling dilution with 0.8
c. of water is made. 2 small drops of 75 per cent arsenic acid are
then added, the solution is heated for 1 to 2 minutes in a boiling
water bath, diluted with 2.6 cc. of water, and the color absorption
determined. The absorption spectrum of the blue compound
developed is given in Fig. 9. The blue color occurs only with
estriol and with no other hormone. The test is about half as
sensitive as the phenolsulfonic acid test (compare Figs. 9 and 1)

![Absorption spectrum](http://www.jbc.org/)

**Fig. 9. Absorption spectrum of the colored compound formed by estriol
in the David test; 32.3 micrograms of crystalline hormone. Compare the
absorption with Fig. 1, middle curve. The ordinate shows extinction
(log (I₀/I)); the abscissa, wave-length in μ.)

but has the advantage of being specific for estriol. Unfortunately
it has proved useless with our urine extracts. Invariably a bluish
cloudy suspension occurs. We have attempted to avoid the
cloudiness by increasing the H₂SO₄ concentration and decreasing
the H₂O, but without success. When the H₂SO₄ is increased to
0.5 cc., the typical blue color does not develop in the extracts.
It is probable that with other methods of extraction or further
purification this test can be extremely useful.

**SUMMARY**

The colorimetric determination of urinary estrin has been
attempted with estrone and estriol fractions of human and rabbit
urines by the use of color absorption methods. The phenolsul-
fonic acid test after the method of Cohen and Marrian (1934)
apparently gives reliable determinations of estriol content in human pregnancy urines of the 6th to 9th months of pregnancy. For the earlier stages of pregnancy it gives a definite overestimate owing to the presence of inactive materials that develop colored compounds absorbing at the wave-lengths of the typical hormone compounds. Estrone fractions appear to contain such materials even in late pregnancy, so that they give an overestimate of hormone content. A rough correction for the inactive materials can be made on the assumption that their colored compounds show a regular increase in absorption from the red to the violet and by subtracting these interpolated absorptions from the total absorption of the extracts. The reacting material in rabbit urines consists practically completely of such physiologically inactive materials, as indicated by the absorption spectra of the treated extracts. The phenolsulfonic acid test as used by Cartland, Meyer, Miller, and Rutz (1935) is somewhat difficult to use with impure extracts, because cloudy solutions form and color fades rapidly.

The benzoyl chloride test distinguishes estrone and estradiol from estriol. It gives a more intense color reaction with estrone than with estradiol. With the estrone fractions of human pregnancy urines it gives hormone values practically identical with those obtained by the phenolsulfonic acid test; with rabbit urine extracts the phenolsulfonic acid titer is doubled.

The David test for estriol is specific for crystalline hormone, but cannot be used with our extracts for quantitative determination because cloudy solutions invariably develop.

We are indebted to Dr. Erwin Schwenk of the Schering Corporation for the crystalline estrone and estradiol used in these experiments and to Dr. Oliver Kamm of Parke, Davis and Company for the crystalline estriol. The bioassays were made by Dr. G. V. Smith and Mrs. O. W. Smith, to whom we express our gratitude.

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