SPECTROFLUORIMETRY OF OESTROGENS:
APPLICATION OF CORRECTION METHODS WHEN
THE UNFILTERED WOLFRAM CONTINUUM
IS USED AS ACTIVATING LIGHT

By

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ABSTRACT

The application of the wolfram continuum as activating light in spectro-fluorimetry of oestrogens has been examined using oestrone as a model substance. When primary filters were omitted, the scatter, deriving from the incident beam, formed a continuous background over which the fluorescence spectra could be recorded in their full extension. In quantitative determinations, fluorescent light could be differentiated by application of correction equations based on recordings of the intensities of narrow spectral bands at the wavelengths of maximal specific emission and at one or two additional wave lengths. When correction methods were to be applied under the conditions examined, the procedure of Ittrich (1958) for colour development and extraction was found to offer the highest sensitivity. The correction equations used in fluorimetry of the Kober colour complex (in methylene chloride) were

\[ F_{\text{corr}} = 2F_{548 \text{ m} \mu} - (F_{518 \text{ m} \mu} + F_{578 \text{ m} \mu}) \]

according to Allen (1950)

or

\[ F_{\text{corr}} = F_{548 \text{ m} \mu} - F_{518 \text{ m} \mu} \]

where \( F \) represents light intensity units. The standard deviation of the corrected blank value in these methods corresponded to less than ±0.2 ng oestrone.

The fluorimetric methods for the estimation of oestrogens offer the advantage of relatively high sensitivity in comparison with the colorimetric methods. The colorimetric methods are generally thought to possess the potentially higher...
specificity since the spectral characteristics of oestrogens in the Kober reaction are favourable for the application of colour correction equations (see Diczfauszy & Lauritzen 1961). The basis for the application of correction equations in colorimetry is provided by the determination of absorption of narrow spectral bands at two or, usually, three regions of wave lengths (Allen 1950), preferably chosen with respect to expected chromogenicity of unspecific material. Two obstacles would be encountered in attempts to introduce analogous measures suitable for the improvement of specificity in fluorimetric methods. Firstly, any reduction of the width of the spectral bands being recorded, leads to impaired sensitivity since such a reduction implies that a smaller fraction of the total emission available is allowed to enter the detector.

Secondly, there are the complications associated with the incident light, part of which is reflected. It is a prerequisite in fluorimetry that reflected incident light be differentiated from the fluorescent light. With respect to efficient activation, it is desirable to use light of wave lengths comprizing those at which maximal absorption occurs.

However, activation at these wave lengths, i.e. at, or close to the peak of the activation spectrum (see Parker & Rees 1962) of the specific substance, is liable to interfere with the recording of the fluorescence spectrum at its shorter wave lengths and even at the wave lengths of maximal emission. This complication derives from the overlapping of the absorption and the fluorescence spectra, the peaks of which, as far as oestrogens are concerned, may occur at wave lengths differing by less than 20 m. In conventional methods, involving determination of emission at one single region of wave lengths, careful selection of filter combinations or monochromator settings has consequently been required in order to exclude scattered incident light from being recorded in addition to the fluorescence.

The present communication deals with the application of an unfiltered continuum as incident light in fluorimetry of oestrogens. Under this condition the reflected incident light forms an internal background which is always recorded in addition to fluorescent light. When present, fluorescent light may be detected by determination of the relative intensities of light at two or three regions of wave lengths. By omitting filtration of the incident light, full advantage is taken of the total absorption capacity of the fluorescing substance and the emission is thus substantially increased. With retention of considerable sensitivity, the entire fluorescence spectrum may thus be resolved by the recording of narrow spectral bands.

In this laboratory the procedure for fluorimetry described above has been used for 2–3 years in combination with very crude as well as more refined methods for the quantitative estimations of oestrogens (e.g. Lunaas 1962, 1963). In the present investigation oestrone has been used as a model substance.

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Fig. 1.
Diagram of the spectrophotometer (Beckman model DU) used for fluorimetry.
View from above.
A. Sample holder in housing lined with felt. The vertical beam of incident light through the cuvette is indicated by $\oplus$.
B. Monochromator.
C. Photocell.
D. Paths of fluorescent light in and out of monochromator.

EXPERIMENTAL AND RESULTS

1. Apparatus
A Beckman model DU monochromator with the commercial photomultiplier combination was used as a fluorimeter. The lamp house was removed and the transmission cell compartment replaced by a metal plate made to form a housing in the rear of the instrument to support a sample holder (Fig. 1).

The sample holder, placed close to the light entrance of the monochromator, was provided with a circular opening (diam., 0.6 cm) at the bottom to allow a vertical beam of incident light to enter the cuvette (Hellma 101 mat – QH, $1 \times 1 \times 4.5$ cm), and a window ($2.2 \times 0.8$ cm) for the fluorescent light. The fluorescent light was thus recorded at a right angle to the incident beam. In order to minimize reflection of incident light, most of the rear wall of the sample holder was cut out. The inside of the supporting house was lined with black felt.

The source of incident light was a vertical projection lamp (Philips 310 G) provided with a collimating lens and a heat filter.

Light intensity was read on the per cent transmission scale as 0-100 or 0-1000 units according to the settings 0.1 or 1.0 of the selector switch. The continuously variable control of sensitivity was always set at the maximum. When needed, the sensitivity was lowered stepwise ($\times 0.281$ and $\times 0.067$) by the photomultiplier control. The phototube load resistor was 100 Mohm.

2. Colour development
In the present study oestrone was used as a model substance. The crystalline steroid (courtesy of Schering AG) was dissolved in ethanol and the solutions dispensed from constriction pipettes into glass tubes ($2.5 \times 15$ cm). For colour development, the Kober reaction of Ittrich (1958) was used with the modification that the oestrone solution (0.2 ml) was taken to dryness together with 0.1 ml of 4% (w/v) quinol in ethanol and
Fig. 2.
Relative fluorescence intensities of oestrone (0.2 µg) developed in 75 % H₂SO₄ and in 52 % of H₂SO₄ and of the Kober colour complex in methylene chloride.
F: Light intensity units.
Incident light: Unfiltered wolfram continuum.
Monochromator slit width: 0.1 mm.
■■■■: 75 % H₂SO₄ (Bauld et al. 1960).
●●●●: 52 % H₂SO₄ (Ittrich 1958), see text.
○○○○: Methylene chloride (Ittrich 1960).

Fig. 3.
A: Absorption spectra of oestrone (20 µg) developed in 75 % H₂SO₄ and in 52 % H₂SO₄ and of the Kober colour complex in methylene chloride. Symbols as in Fig. 2.
E: Optical density.
B: Relative intensities of the unfiltered wolfram continuum (upper curve) and of the light transmitted by four different filters. Recording of reflection from white paper. Monochromator slit width: 0.1 mm. Note that the highest intensities of the continuum occurred in the region of wave lengths at which the Kober colour complex in methylene chloride had its maximal absorption capacity.
the residue treated with 4 ml of 52% (v/v) sulphuric acid (Merck AG) to which no quinol had previously been added. The tubes were closed with glass cones and held in the boiling water bath for 40 minutes. The sulphuric acid was subsequently cooled and diluted with 5 ml of water, cooled again and extracted with 4 ml methylene chloride containing 2% (w/v) p-nitrophenol (Ittrich 1960).

Oestrogen was also developed in 75% (v/v) sulphuric acid for 10 minutes according to Bauld et al. (1960).

3. Fluorescence of oestrogen in different media

In Figs. 2 and 3 are recorded the fluorescence and absorption spectra of oestrogen developed in 75% H₂SO₄ and in 52% H₂SO₄ in the presence of quinol and of the Kober colour complex in methylene chloride. The fluorescence spectra, extending from 450 mµ to 600 mµ, were recorded with the slit width fixed at 0.1 mm. No attempts were made to correct the spectra for variations in the width of the spectral bands recorded or in the sensitivity of the detector. It may be seen that the highest fluorescence intensities were obtained in methylene chloride in which the Kober colour complex had its peak absorption at 532 mµ. At this region of wave lengths the incident light appeared to have its highest intensities (see Fig. 3), the maximal values being recorded at 530 mµ. The intensities of the incident light at 510-520 mµ, i.e. at the wave lengths of peak absorption of the Kober colour in 52% H₂SO₄ (516 mµ), were about 95% of the maximal value. The fluorescence in the Kober reagent, however, still amounted only to 1/3 - 1/2 of that in the methylene chloride. In this case the intensity of the fluorescent light was evidently dependent on the nature of the medium.

The apparent intensity of the fluorescence in 75% H₂SO₄ was about 3/4 of that in the Kober reagent. The apparent intensities of the incident light at the wave lengths of maximal absorption in these media were about 1:2. The ratio between emitted and absorbed light thus seemed to be lowest in the Kober reagent.

4. Filtered v. unfiltered light for activation

In Table 1 (cf. also Fig. 3 B) are recorded the characteristics of some filters (Schott) which were inserted in the path of incident light. The filters were examined with the fluorimeter using white paper as a reflector. The slit width used was 0.1 mm which corresponded to a total band width of 5-6 mµ at the 546.1 mµ Hg line. The maximal transmission of the filters amounted to about 40%. The spectral bands transmitted were rather narrow, the half intensity widths ranging from 12 to 20 mµ. It was found that by filtration of the incident light to obtain spectral bands for activation, the fluorescence was lowered to 1/10 or less of that obtained with the unfiltered continuum. In order to compensate for the loss of sensitivity due to the primary filters transmitting maximally at 495, 509 and 522 mµ it was necessary to record the fluorescence over 3-5 times broader bands at the wave lengths of maximal emission. More suitable filters could conceivably have been found for the purpose of activation in the particular situation selected (cf. the shape and the position of the absorption spectrum of oestrogen in the Kober reagent and the characteristics of the different filters, Fig. 8). However, the example chosen serves to demonstrate that any filtration of the incident light is likely to reduce the fluorescence seriously, the reason being that a much smaller fraction of the light from the source applied can then be absorbed.

5. Methods of correction for reflected incident light

From Fig. 3 it may be seen that the apparent intensity of the incident light in-
Table 1.
Comparison between unfiltered and filtered light for the activation of the Kober colour of oestrone (0.2 µg) in 52% H₂SO₄. Maximal absorption and fluorescence at 516 mµ and 538 mµ respectively (cf. Figs. 2 and 3).

<table>
<thead>
<tr>
<th>Characteristics of filter:</th>
<th>No filter</th>
<th>Filter A</th>
<th>Filter B</th>
<th>Filter C</th>
<th>Filter D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wave length of maximal transmission, mµ</td>
<td>495</td>
<td>509</td>
<td>522</td>
<td>543</td>
<td></td>
</tr>
<tr>
<td>Half intensity band width, mµ</td>
<td>14</td>
<td>16</td>
<td>12</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Transmission at maximum, per cent</td>
<td>37</td>
<td>42</td>
<td>45</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Light intensity recorded at 538 mµ, relative values</td>
<td>100</td>
<td>4.8</td>
<td>10.9</td>
<td>10.6</td>
<td>4.5</td>
</tr>
<tr>
<td>Width of slit required for compensation, mm</td>
<td>0.1</td>
<td>0.51</td>
<td>0.34</td>
<td>0.32</td>
<td>0.58</td>
</tr>
<tr>
<td>Corresponding total band width as estimated at the 546.1 mµ Hg line, mµ</td>
<td>5-6</td>
<td>25</td>
<td>18</td>
<td>16</td>
<td>29</td>
</tr>
<tr>
<td>Reflected light in per cent of total light recorded at 538 mµ</td>
<td>8</td>
<td>2.6</td>
<td>1.7</td>
<td>2.1</td>
<td>59</td>
</tr>
</tbody>
</table>

creased almost linearly with the wave lengths in the region 450-520 mµ which is the region of wave lengths in which the fluorescence of oestrone occurs in 75% H₂SO₄. The interference of incident light in the determination of fluorescence could therefore be minimized by reading at three wave lengths and application of a correction equation analogous to those introduced in the colorimetry of steroids by Allen (1950). In Table 2 are recorded the absolute values obtained of light intensity units read at the wave lengths of maximal fluorescence and also the values calculated on the basis of readings at the maximum and at 50 mµ to each side. The method of correction led to a reduction of the ratio between the background and the fluorescence of 0.2 µg oestrone from about 1 : 10 to 1 : 1000.

Due to the convex shape of the background curve at 510-600 mµ, corrections based on readings at three wave lengths were less efficient in this region than at 450-520 mµ. However, the calculated values of the background at the wave lengths of maximal fluorescence of the Kober colour complex in methylene chloride (548 mµ) were considerably smaller than the absolute values recorded and corresponded to about 0.001 ± 0.0002 µg oestrone.

In Table 2 the values obtained by subtracting the light intensity units recorded at 518 mµ from those recorded at 548 mµ are also given. At these wave lengths the intensities of the incident light amounted to 98 and 94 per cent respectively of the maximal intensity which was recorded at 580 mµ (cf. Fig. 3). The correction thus resulted in a negative value for the reagent blank. Zero values could be obtained by reading at 514 mµ, at which wave length the intensity of the reflected light equaled that at 548 mµ.

The maximal emission of the Kober colour complex of 3-methyl-oestrone in methylene chloride occurred at 549-550 mµ.
Table 2.

Absolute and corrected light intensity values (means ± SD, n = 6) of oestrone in different media and of reagent blanks. Incident light: Unfiltered wolfram continuum. Monochromator slit width: 0.1 mm.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Oestrone, µg</th>
<th>Light intensity units</th>
<th>Correction equation, ( F_{corr} = )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Absolute values*(^{a})</td>
<td>Corrected values, ( F_{corr} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reagent + oestrone</td>
<td>Reagent blank</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>75% H₂SO₄</td>
<td>0.200</td>
<td>332.8 ± 14.2</td>
<td>31.7 ± 5.1</td>
</tr>
<tr>
<td>Kober reagent</td>
<td>0.200</td>
<td>481.7 ± 10.3</td>
<td>38.3 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>0.200</td>
<td>1127.5 ± 78.9</td>
<td>37.3 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>0.020</td>
<td>153.3 ± 4.1</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>47.2 ± 2.1</td>
<td>---</td>
</tr>
<tr>
<td>Methylene chloride</td>
<td>0.200</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.020</td>
<td>106.7 ± 3.3</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>8.0 ± 0.6</td>
<td>---</td>
</tr>
</tbody>
</table>

*\(^{a}\)) Recorded at the wave length of maximal fluorescence.
Table 3.
The blank values and their estimated standard deviations in three different methods for the fluorimetric determination of oestrogens involving extraction of the Kober colour according to Ittrich (1958).

<table>
<thead>
<tr>
<th>Method</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation of activating light</td>
<td>Monochromator</td>
<td>Filters</td>
<td>Omitted</td>
</tr>
<tr>
<td>Isolation of fluorescent light</td>
<td>Monochromator</td>
<td>Filter</td>
<td>Monochromator</td>
</tr>
<tr>
<td>Width of band recorded of fluorescent light, µµ</td>
<td>5 and 20</td>
<td>50</td>
<td>5 – 6</td>
</tr>
<tr>
<td>Amounts of oestrone corresponding to the blank value ± SD, ng</td>
<td>9.17 ± 2.30</td>
<td>0.78 ± 0.13</td>
<td>a: 0.94 ± 0.15, b: 0.07 ± 0.12</td>
</tr>
</tbody>
</table>

Method I: Støa & Thorsen (1962).
II: Roy (1962).
III: Present study.

Equations for the calculation of corrected light intensity values in method III (cf. Table 2):

a: $F_{corr} = 2F_{548} - (F_{518} + F_{578})$,
b: $F_{corr} = F_{548} - F_{518}$.

DISCUSSION

The highest specificity in fluorimetry can be expected when activating with narrow spectral bands at the peak of the absorption spectrum and simultaneously recording the fluorescence at the wave lengths of maximal emission. In the method proposed in the present paper the former condition is not satisfied. Thus, as far as specificity is concerned, the disadvantage of the procedure examined is obviously that fluorogenic impurities having spectral characteristics other than those of the oestrogens, may also be activated and contribute more to the fluorescence than when the activating light is rigorously selected with respect to the specific substance. It was shown that activation with narrow spectral bands, i.e. small fractions of the full continuum, yields relatively weak fluorescence. It is apparent therefore that when both of the two conditions stated to be preferable with respect to specificity are to be satisfied, decreased sensitivity must be accepted. The accuracy in fluorimetry becomes dependent on inner filter effects when coloured material occurs in the medium in which the fluorescence is measured. In such cases, for example, in simple techniques for the estimation of oestrogens in urine, the accuracy may be improved if the sensitivity of the method for fluorescence determina-
tion allows of higher dilutions. It is of interest to evaluate the sensitivity obtainable when applying the correction procedures proposed, in connection with activation with the full continuum. According to Wilson (1961) the sensitivity of a method is to a large extent governed by the variation of the blank value. In Table 3 are listed the blank values and their estimated standard deviations in two different fluorimetric methods recently published and in the method presented in this paper. The values have been calculated as oestrone equivalents for the sake of comparison. The method of Støa & Thorsen (1962) presumably possesses a high degree of specificity since monochromators were used for the isolation of incident as well as fluorescent light in agreement with the conditions mentioned above. Roy (1962) applied a filter combination and recorded a rather broad band of the fluorescent light. By this method, as judged from the standard deviations of the blank values, a far higher sensitivity was attained than with the monochromator instrument, operated to yield relatively narrow spectral bands. It may be seen that the sensitivity when introducing corrections for reflected incident light, is comparable to that obtained by Roy (1962) although the spectral bands recorded of the fluorescent light were far smaller in width in the present method, namely 5–6 mμ as compared to 50 mμ.

It is evident that under the conditions described, the extraction procedure of Ittrich (1958, 1960) offered the highest sensitivity. In the present study the readings required for the application of correction equations were taken at wave lengths differing by 30 mμ from those at which maximal fluorescence occurred. However, the shape of the emission spectrum of the Kober colour complex in methylene chloride is such, that the readings may be taken closer to the maximum without very serious loss of sensitivity. This possibility might be considered when interference unspecific fluorescence is expected. Variation in the widths of the spectral bands recorded would not lead to material alterations in the ratio between fluorescent and reflected light. Thus at the risk of loss of specificity due to poorer spectral resolution, a wider slit may be used, when the signal-to-noise ratio of the detector system is the factor limiting the sensitivity.

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REFERENCES


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