UV Spectrophotometry in Medium-Band Instruments

To the Editor:
In a recent Letter to the Editor entitled “A Common Error in Measurements at 340 nm,” it was reported that the molar absorptivity (ε) of NADH is significantly lower in medium-band than in narrow-band spectrophotometers, and that medium-band instruments should be calibrated with an NADH standard if significant errors are to be avoided (1). These conclusions were based on absorbance measurements of NADH solutions in a Zeiss PMQ II (1.5-nm bandwidth) and a Gilford 300-N (8-nm bandwidth) spectrophotometer. The ε value for NADH in the Gilford 300-N was, on the average, 94.5% of that obtained in the Zeiss PMQ II. We wish to present evidence that this discrepancy cannot be attributed solely to the difference in spectral bandwidth of the two instruments. Such a discrepancy can also arise from other factors that affect photometric accuracy such as light path of the cuvettes, stray light, wavelength accuracy, etc. (2, 3).

Examination of the spectrum of NADH in the region of 340 nm shows a broad absorption maximum at 337–339 nm, and an average absorbance from 335–345 nm equal to 99.3% of that at 340 nm (4). A medium-band spectrophotometer (8–10 nm) therefore could introduce an error of less than 1% as compared with the 5.5% reported (1). This calculated small effect of bandwidth on the absorbance of NADH was confirmed as follows: A solution of NADH (1.83 × 10^{-4} mol/liter, 1Cat. No. 2200; P-L Laboratories, Milwaukee, Wis. 53233) was prepared by dissolving 159 mg in one liter of 0.1 mol/liter phosphate buffer, pH 7.5, and its absorbance was measured in a Beckman DU spectrophotometer from 320–357 nm, with bandwidths of 1.0 and 10 nm. Figure 1 shows that the two absorption curves are superimposable. A similar observation was made with a Cary 16 spectrophotometer. Absorption spectra of solutions of NADH and K_{2}Cr_{2}O_{7}, with a peak absorbance near 1, were obtained for bandwidths of 1.5 nm and 8 nm. With the 8-nm bandwidth, the absorbance of both solutions was 99% of that obtained with the 1.5-nm bandwidth.

Because the difference in ε values for NADH observed by Schales (1) could also be attributed to variations in the light path of the cuvettes, this source of error was investigated. Solutions of NADH are unstable (6), and a reference sample of known purity is not available. For these reasons, we used National Bureau of Standards (NBS) certified potassium dichromate to test the photometric accuracy of the Gilford 300-N spectrophotometers in our laboratories. Potassium dichromate exhibits a broad maximum at about 350 nm, ε = 3.14 ± 0.04 × 10^5 in 0.01 mol/liter H_2SO_4 (2) and 3.16 ± 0.02 × 10^5 in 1 mmol/liter perchloric acid (3). The latter value was used for our studies. Table 1 shows the variation in absorbance obtained for seven Gilford 300-N instruments. Although each 300-N was calibrated with the absorbance filter (A_{550}) provided by the manufacturer, considerable variation was observed, 95.9 to 101.5% of the expected absorbance. The possibility that this variation was due to differences in the light path of the cuvettes was excluded by the fact that the absorbance value for each instrument remained practically the same when a single cuvet was used in all seven instruments.

The above results clearly show that neither bandwidth nor light path can account for the variation in photometric accuracy obtained with the Gilford 300-N spectrophotometer (Table 1). In our experience a variety of medium-priced (and occasionally even expensive) spectrophotometers exhibit similar absorbance errors. We fully agree with Schales (1) that one must be aware of this problem and attempt to correct it by recalibration.

Morell et al. (7) used NBS certified potassium dichromate to calibrate the Gilford 300-N. An error or change in the absorbance calibration filter provided with each instrument is probably a principal source of error, which can easily be corrected as illustrated in the following example: Instrument No. 2159 exhibited a higher

![Fig. 1. Absorbance of NADH at narrow and wide bandwidths](image)

**Table 1. Absorbance of NBS-Certified Potassium Dichromate** in Several Gilford 300-N Spectrophotometers and in a Cary 16

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Absorbance at 350 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gilford 300-N, serial no.</td>
<td>A_{550} filter setting</td>
</tr>
<tr>
<td>3812</td>
<td>.955</td>
</tr>
<tr>
<td>3813</td>
<td>.965</td>
</tr>
<tr>
<td>3814</td>
<td>.973</td>
</tr>
<tr>
<td>3815</td>
<td>.993</td>
</tr>
<tr>
<td>2159</td>
<td>.951</td>
</tr>
<tr>
<td>2915</td>
<td>.967</td>
</tr>
<tr>
<td>3659</td>
<td>.977</td>
</tr>
<tr>
<td>Cary 16</td>
<td>1.073</td>
</tr>
</tbody>
</table>

^{a} 9.40 × 10^{-4} mol/liter (100.0 mg/liter) in 10^{-3} mol/liter perchloric acid (3).

^{b} Calculated from ε = 3.16 ± 0.02 × 10^{5} (3).

^{1} Calculated from ε_{550} = 107 ± 1 (2).

^{2} Calculated from 2 × ε reported for the monobasic anion, (HCrO_{4})^{-}, in a K_{2}Cr_{2}O_{7} concentration range of 20 to 100 mg/liter (Ref. 3, Tables 6 and 7) as suggested by Dr. R.T. Burke, National Bureau of Standards (personal communication). The ε value, 3.16 ± 0.02, is applicable only to the specific conditions used, 20 to 100 mg/liter NBS certified K_{2}Cr_{2}O_{7} in 10^{-3} mol/liter HClO_{4} at 25°C, the temperature dependence being −0.05% per degree from 17 to 37°C (3).
Radioassay of Folate

To the Editor:

Dr. Shaw, in a recent issue of Clinical Chemistry (19, 281, 1973), raised some questions about a radioassay method that we recently published elsewhere (1), and we now wish to answer each comment point by point.

1. The description of the steps for purifying the folate binder from milk was complicated by the omission of an important sentence that was corrected in a later issue of that journal.

2. The measurement of folate concentration in the milk solution was done by spectrophotometry at 340 nm.

3. We used a Gilford spectrophotometer with a filter of 0.850 nm.

4. The extinction coefficient of the reduced band of pyridine nucleotides is 175385 (1948).


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Fig. 2. Effect of A550 filter calibration on A550 of NBS certified potassium dichromate, 3.603 × 10^-4 mol/liter

than expected absorbance at the original filter setting of 0.951 (Table I). Using a 3.40 × 10^-4 mol/liter solution of K2Cr2O7, we realigned the A550 absorbance calibration setting to 0.9, to obtain the expected absorbance of 1.074. Figure 2 (center line) shows the linear relationship between absorbance and K2Cr2O7 concentration for the adjusted setting. Linearity prevailed even when the setting was increased or decreased by 0.05 absorbance units.

In conclusion, we suggest that in absorbance measurements where NADH is produced or consumed, the spectrophotometer in use be calibrated with NBS-certified K2Cr2O7 dissolved in 10^-3 mol/liter perchloric acid. Potassium dichromate is available in very pure form, its molar absorptivity is well established, and solutions in perchloric acid are very stable (2, 3).

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References

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