THE ADAPTATION OF THE BECKMAN SPECTROPHOTOMETER TO MEASUREMENTS ON MINUTE QUANTITIES OF BIOLOGICAL MATERIALS

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The smallest volume that has been used with commercial photocolorimeters is about 1 ml. The sensitivity and optical system of the Beckman spectrophotometer (model D or DU) are such that it can be readily adapted to volumes of the order of 50 c.mm. (0.05 ml.) without sacrificing length of the light path through the colored solution. With volumes of this order of magnitude the size of sample required is so small that it has been easily possible to devise methods for determining a number of substances of nutritional significance on a single sample of finger blood (e.g. vitamin A, carotene, ascorbic acid). Since it seems likely that colorimetry or spectrophotometry on this scale may prove useful on other occasions, particularly with biological material, when the size of sample is limited, the means of adaptation is described, together with some data illustrative of the precision which can be expected.

Principle

Samples are placed in a special cuvette (Fig. 1) which has the usual 1 cm. light path but which has a narrow inner width, of 2 mm. or less. 50 c.mm. of liquid will fill this cuvette to a height of about 2.5 mm., thus giving a column of the sample with a cross-sectional area of 2 × 2.5 mm. and a length of 1 cm. In front of the cuvette is placed a diaphragm which confines the light beam to a cross-section of less than 2 × 2 mm. This slender light beam can pass through the liquid without touching the cuvette walls or the liquid meniscus. In order to obtain sufficient light intensity for measurements when such a small area of the photocell is used, it is usually necessary to widen the spectral band employed; however, the band widths used are still narrow in comparison with those of most other photoelectric spectrophotometers or photocolorimeters. Alternatively, the use of a more sensitive galvanometer permits the use of narrower spectral widths than would otherwise be necessary.

Equipment

Cuvettes—These are square cells having inner dimensions of 2 × 10 mm. instead of the usual 10 × 10 mm. and are only 25 mm. high (Fig. 1).
Except for the height, the outer dimensions are the same as those of the standard cuvette. The light path is 10 mm., the same as for the regular cuvettes; however, the cross-sectional area is greatly reduced. (These cuvettes may be obtained in quartz from the Pyrocell Manufacturing Company, 207 East 84 Street, New York 28, New York.)

Smaller cuvettes (1 × 10 mm. inner dimension) requiring 30 c.mm. of liquid have also been used successfully, but, since their use is a little more difficult, it is recommended that the larger (2 mm.) cuvettes be given a trial before attempting to use the smaller ones.

Diaphragm—Two types (A and B) have been used and both have been found to be satisfactory. Type A can be home-made (Fig. 1). A piece of metal of the size and shape of a penny is used. This must just fit the opening in the instrument, which leads the light beam toward the cuvette holder. A 1.0 to 1.4 mm. hole is drilled through the metal disk about 1 mm. off center. The disk is then held in the opening with a piece of Scotch tape and turned until the emergent pencil of light passes exactly in the middle between the two walls of the cuvette when in place in the carriage. The disk is now fastened at just this angle to a very flat thin piece of sheet steel. This sheet is about 6 cm. wide by 9 cm. long, with a 3 or 4 mm. hole 2.5 cm. from one end. The disk is soldered to the sheet so that the holes coincide and the long axis of the sheet is perpendicular. After soldering, the top of the sheet is bent at a right angle to form a flange which lies on the top of the instrument and keeps the sheet from turning. The cuvettes are now raised
on wooden blocks (Fig. 1) until the light beam just misses the bottom of the cuvette. (Special adjustable blocks may be obtained from the Pyrocell Manufacturing Company.) As much as possible of the “play” in the cuvette carriage assembly is taken out. The diaphragm may be inserted and removed by merely loosening the bolts holding the phototube housing. The cuvettes are ordinarily held close to one side of the carriage by a spring on the opposite side. When used for the micro cells and when the diaphragm is being adjusted, the carriage should be oriented so as to bring the cuvettes as near the diaphragm as possible, since the light beam spreads out a little after leaving the diaphragm, and the closer the cuvettes are to the diaphragm the less difference this will cause. The cuvettes are numbered and always inserted in the holder with the same orientation.

Type B, a somewhat more convenient and easily adjustable diaphragm attachment, may be obtained from the Pyrocell Manufacturing Company. The pinhole or pinholes are carried by a thin strip of brass (Fig. 1) which slides in a channel cut in a sheet of metal which is inserted in the spectrophotometer between the cuvette carriage assembly and the body of the instrument. The sheet of metal is held in exact position by holes drilled through it to match the precision pins of the instrument. After the metal sheet is inserted, the brass strip is brought to position by sliding it into the channel in the metal sheet until the pinhole coincides with the center of the cuvette. A stop on the brass strip is then adjusted with a special bolt so that the strip may always be brought to exactly this same position. Provision is made so that either of two or three different sized pinholes may be brought into position without disturbing this adjustment. With this diaphragm the cells are raised on blocks, as described above. With the strip removed the instrument may be used with standard cuvettes without disturbing the metal sheet.

Procedure

Since there is usually a little “play” in the cuvette carriage, the cells should always be brought toward their position in front of the light from the same direction. If there are any traces of cement remaining on the cuvette walls, these should be removed with a needle, etc. The micro cuvettes are used like the larger cells except that, for convenience in handling, they are left mounted in the carriage. Samples are introduced with fine tipped pipettes into the bottom of the cuvettes, and the previous samples are removed by suction with a fine tipped glass tube attached to a suction pump or aspirator bottle. If the glass tip is slender, 99 per cent of the previous sample may be removed, thus eliminating the need for rinsing the cuvettes between analyses of a series unless the concentrations are widely different.
The 2 mm. cells require about 50 c.mm. of solution. The exact amount necessary should be determined by pipetting in solution until further addition is without effect. A large cell may be used in the first position in the holder, which is merely for adjusting the instrument to zero for the particular solvent or other blank being used. The small cells should give blank readings that differ by no more than about 0.01 on the density scale from the first (large) cell, and these blank readings should be reproducible to within 0.002 on the density scale.

### Table I

**Optical Density of Replicate Samples of Different Volumes Taken from Same Large Volumes**

<table>
<thead>
<tr>
<th>0.0465 mg. per cent P</th>
<th>0.0186 mg. per cent P</th>
<th>0.00465 mg. per cent P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025 ml. 0.05 ml. 3 ml.</td>
<td>0.025 ml. 0.05 ml. 3 ml.</td>
<td>0.025 ml. 0.05 ml. 3 ml.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mg. P x 10^-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.6 23.2 1390 4.65 9.3 560 1.16 2.32 139</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Optical density (870 m(\mu))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.420 0.423 0.424 0.168 0.167 0.166 0.043 0.040 0.042</td>
</tr>
<tr>
<td>0.420 0.422 0.424 0.166 0.165 0.166 0.043 0.043 0.043</td>
</tr>
<tr>
<td>0.420 0.422 0.424 0.166 0.166 0.166 0.042 0.042 0.042</td>
</tr>
<tr>
<td>0.420 0.422 0.420 0.167 0.167 0.165 0.043 0.043 0.042</td>
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<tr>
<td>0.425 0.420 0.424 0.420</td>
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<tr>
<td>0.422 0.423 0.424 0.423</td>
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<td>0.421 0.421 0.424 0.424</td>
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<tr>
<td>0.419 0.421</td>
</tr>
<tr>
<td>0.422 0.422</td>
</tr>
</tbody>
</table>

| Average | 0.421 0.422 0.423 0.167 0.166 0.166 0.043 0.043 0.042 |
| S.D. | 0.0017 0.0009 0.0014 0.0009 0.0010 0.0007 0.0005 0.0019 0.0004 |
| Variance, % | 0.4 0.2 0.3 0.5 0.6 0.4 1 4 1 |

With the early Beckman instruments a more sensitive galvanometer has been found to be advantageous for use with the micro attachment. The meter furnished with the instrument is replaced by one with a range of 200 microamperes. This is 5 or 10 times more sensitive than the original meter and has proved useful for macro as well as micro work. The sensitive meter is particularly valuable for use with the 1 mm. cuvettes or for measurements with larger cuvettes at wave-lengths at which otherwise insufficient light is obtained. Under these circumstances, it is sometimes necessary to adjust the instrument to zero at the “0.1” knob position. This allows the instrument to be used with one-tenth the ordinary amount of light, and, with the more sensitive meter, readings may be made as ac-
accurately at this setting as when the instrument is adjusted to zero with the
"check" position of the knob. With later instruments the original sensitivity is such that a more sensitive meter is not required.

DISCUSSION

Table I illustrates readings obtained at 870 m\(\mu\) with 3, 0.05, and 0.025 ml. of solutions taken from three large volumes of inorganic phosphate

Fig. 2 gives the absorption curves obtained with the strongest of the above phosphate solutions (0.0465 mg. per cent). One curve was obtained

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**Diagram:**

- **Fig. 2.** Absorption curves of phosphomolybdous acid with 3 and 0.025 ml. volumes of different strengths treated to form the blue phosphomolybdous color. (The color was developed with a modified Fiske and Subbarow reagent, 2.5 per cent ammonium molybdate, 0.6 per cent sodium bisulfite, 0.03 per cent sodium sulfite, and 4.5 mg. per cent of 1,4-aminonaphtholsulfonic acid in 1.2 N sulfuric acid, by heating 20 minutes at 100\(^\circ\).)
with 3 ml. of solution with the narrowest slits possible. The other curve was made with 0.025 ml. of solution (10⁻³ mg. of P) and necessarily a wider slit. The curves are so nearly superimposable that the second set of points was not connected in order to avoid confusion.

Fig. 3 gives a similar set of curves made with phenylalanine in the ultraviolet region. This compound was chosen since its spectrum contains a series of very narrow absorption bands. In this case the curves obtained

![Fig. 3. Absorption curves of phenylalanine with 3, 0.05, and 0.025 ml. volumes](image)

with 3, 0.05, and 0.025 ml. of solution are not superimposable, since with the wider slits required for the small volumes some of the fine detail is obscured. However, the difference between the 3 ml. and the 0.05 ml. curves is not marked. This illustrates a limitation which should be borne in mind if spectra with fine structure are to be measured. This difficulty, of course, would not interfere with quantitative measurements made even on compounds such as phenylalanine.
It is evident that satisfactory results may be obtained with the small volumes for both quantitative measurements and absorption curves. The 2 mm. cuvettes have proved to be as convenient to use in practice as the larger cells. Measurements may be made even more rapidly with the 50 c.mm. volumes than with larger samples.

Spectral curves can be made from about 235 to 935 μm with spectral widths not exceeding 3 μm with 25 c.mm. volumes, and from about 225 to 1050 μm with spectral widths of not more than 3 μm with 50 c.mm. volumes. With the type of adaptation described, it does not appear possible to utilize much smaller volumes than 25 c.mm., since with still narrower cells the setting of the carriage position would become too critical. It will probably be necessary to employ instead some other principle for adaptation to lesser volumes.

It has been felt desirable to use a fixed diaphragm with these cells instead of providing each cell with its own diaphragm which would then move with the cell. The disadvantage of such a movable diaphragm would be that a more refined carriage movement would be required. The light beam differs in brightness from point to point, as does the sensitivity of the phototube surface; hence minute differences in the point at which the carriage comes to rest would affect the phototube response.

With the adaptation described it has been possible to devise methods for the measurement of ascorbic acid in 0.01 ml. of serum,1 vitamin A and carotene in 0.035 to 0.06 ml. of serum,2 and the ascorbic acid in the white blood cells and platelets of 0.1 ml. of blood.3

**SUMMARY**

A description is given for the adaptation of the Beckman spectrophotometer to the performance of colorimetry and spectrophotometry on volumes of 0.05 ml. or less. Illustrative data and absorption curves are included. Thus adapted, the measurements on volumes of this order of magnitude are as rapid and convenient and very nearly as precise as with larger volumes.

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