Micromethods for the Clinical and Biochemical Laboratory

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Foreword

In recent years there has been an avalanche of techniques and instruments available for the analysis of components of interest to the biochemist and clinical chemist. Each time a new technique is made known, numerous papers appear describing modifications to it, and the laboratory worker who desires to use these methods is faced with the difficult choice of which variant to use. Dr. Mattenheimer has performed a most useful service for us in this volume by describing in detail the instrumental and methodological requirements for proper microchemical analyses. The methods presented have been thoroughly tested and their reliability and accuracy proven by daily use. Even the busiest of laboratories will find these microliter methods advantageous for many of their high volume routine procedures. The obvious savings in time, space and money of micromethods are of concern to us all.

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Preface

About forty years ago the development of biochemical micro- and ultramicromethods began at Carlsberg Laboratory, Copenhagen. In the hands of Linderstrøm-Lang and H. Holter and their many co-workers and students, this new approach opened the way for biochemical studies on minute cell, tissue and fluid samples.

From 1955–57 I received my training in micromethodology in Dr. Holter's department at Carlsberg Laboratory. Although my research project was the enzymology of amoeba, my interest grew in the development of micromethods in clinical chemistry. Of the various approaches possible for the development of micromethods, I chose, whenever possible, the proportional reduction of sample and reagent volumes. In this way it was possible to convert widely accepted and well tested conventional macromethods into micromethods—or microliter methods, to use a more modern term. The application of micromethods to routine clinical chemistry had to await the commercial manufacture of the necessary special equipment, which is now available.

A limited collection of micromethods was published in the first German edition of this book in 1961. The wide acceptance and favorable reviews of the book led to the revised and substantially enlarged second German edition in 1966, which covers the almost complete line of chemical and enzymatic methods requested from a modern clinical laboratory.

The first American edition is a translation of the second German edition with only a few revisions necessitated by the differences in the market for equipment and reagents between Europe and this country. In addition a few of the methods have been substituted for procedures more commonly used in the United States. With a few exceptions the micromodifications described in this book were worked out in my laboratory. The methods that were originally published as micromethods are marked with *micromethod* behind the literature reference.

The content of this book is not limited to routine clinical chemistry but includes techniques for biochemical research with a special chapter on ultramicrotechniques, which were mainly developed by O. H. Lowry and are suitable to quantitatively measure enzyme activity in a few cells dissected from frozen-dried tissue sections.

The research in my laboratory is supported by grants from the United States Public Health Service.

Chicago, Illinois
April, 1970

Hermann Mattenheimer

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Chapter 1

Micromethods: Definition and Experience

The term micromethod is ambiguous and can be used to describe several methods:

1. It refers to a method which detects a substance present in very small amounts in a relatively large volume. The prefix "micro" denotes the concentration. To detect the substance in question, either it must be enriched or extremely sensitive techniques must be employed.
2. It refers to a method which analyzes a small volume for a substance present in a relatively high concentration. The prefix "micro" denotes the volume. If the volume is between 10 μl and 100 μl (1 μl = 0.001 ml) the method is called a *microliter method,* a term introduced only recently. All analytical procedures in Chapters 4–7 of this book belong to this group of tests.
3. It refers to a method which analyzes a small volume for a substance present in a relatively low concentration. The prefix "micro" denotes the volume as well as the concentration. Depending on the size of the volume such a method may still be a microliter method, but with volumes of less than 10 μl, down to a fraction of a microliter, one speaks of an ultramicromethod (Chapter 8). For example, ultramicro analytical procedures include fluorometry.

The number and variety of tests have increased substantially during the past 15 years, and a request for five or more analyses on a single blood sample of a patient today is about an average figure. Most macromethods require 1 ml of serum, and for five tests done in duplicate this comes to 10 ml serum or 20 ml blood. Repeated withdrawal of such quantities, particularly from severely sick patients, often becomes undesirable.

Considering the number of tests performed on infants' blood it is not surprising that pediatricians urge the use of microliter methods. For microliter methods, the chemist needs at most one-tenth of the volume ordinarily required for macromethods; thus five or more tests can be performed on 1 ml of serum.

Microliter methods have several advantages for the routine clinical and research laboratory, the most important of which are saving of time, money, and space. The chemist who uses microliter methods in his laboratory knows that an analysis can often be performed in one-half of the time required for macroprocedures. Less space is needed for equipment because it can conveniently be placed on the laboratory bench within reach of the technician.

Most microcentrifuges are capable of speeds up to 20,000 rpm with centrifugal forces of about 25,000 g. They accelerate to maximum speed within 15–30 seconds and need little time to stop; this cuts the time for centrifugation to a few minutes. Pipetting of small volumes, especially with constriction pipets, is several times faster than pipetting of ml quantities. An outsider may not understand these considerations, but time saving is a decisive factor considering the workload in a routine laboratory.

Micromethods save chemicals. Modern analytical techniques require expensive biochemicals such as enzymes, substrates and coenzymes; a substantial relief in the budget is felt when only one tenth of the otherwise necessary quantities is consumed. In increasing numbers clinical laboratories introduce microliter procedures into their routine program and several perform microliter methods exclusively.

The accuracy of a microliter method is equal to the accuracy of the macromethod from which it was adapted. The experimental error of methods in the clinical laboratory is usually between 0.5–5%. Experience in our laboratory has shown that, even with ultramicro procedures which include the weighing of tissue samples in mμg-quantities, the coefficient of variation rarely exceeds 5%. The error of the chemical analysis *per se* is not greater than 2–3%.

Thorough training in microliter methods is of course essential. A capable technician can learn microanalysis within three to four weeks.

Only a few technicians and academically trained chemists do not develop the skill for these methods. With the techniques mastered, the results of the analyses will depend mainly on the quality of the technical tools.

Until a few years ago it was left to the chemist to develop his own or to modify existing instruments and tools for microanalysis. The work of the pioneers in this field, H. Holter, O. H. Lowry, D. Glick and P. Kirk (to name a few) was the guidepost for most developments. Industry has now caught up with the many requests for special instrumentation and most of the equipment essential to the microanalytical laboratory is now commercially available.

Chapter 2

Equipment for Microliter Analysis

PHOTOMETERS

Photometric measurements with microcuvettes require a photometer with an adapter to confine the light beam to a cross-section narrow enough to pass through the liquid without touching the walls of the cuvette or the meniscus of the fluid. This is achieved by a simple pinhole or slit diaphragm*, or, to avoid a substantial loss of light, by an optical system.

A number of spectrophotometers and filter photometers with accessories for microliter analysis are commercially available, and their discussion is based on the author's personal experience.

Zeiss spectrophotometer PMQII

A special sample changer for microliter cuvettes with a built-in optical system (Figure 2) is available for this instrument (Figure 1). The optical system permits most of the light issuing from the monochromator to traverse tubular microcells up to a length of 5 cm. The spectral band

* Lowry, O. H., J. Biol. Chem. **163**, 633 (1946).

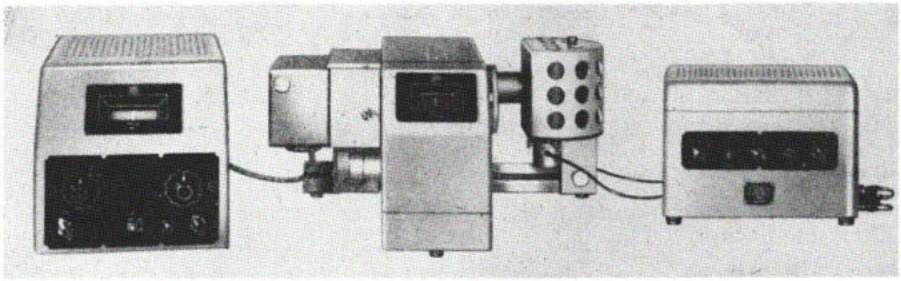

Figure 1. Zeiss Spectrophotometer PMQ II (Carl Zeiss, West Germany, Carl Zeiss, Inc., New York, New York).

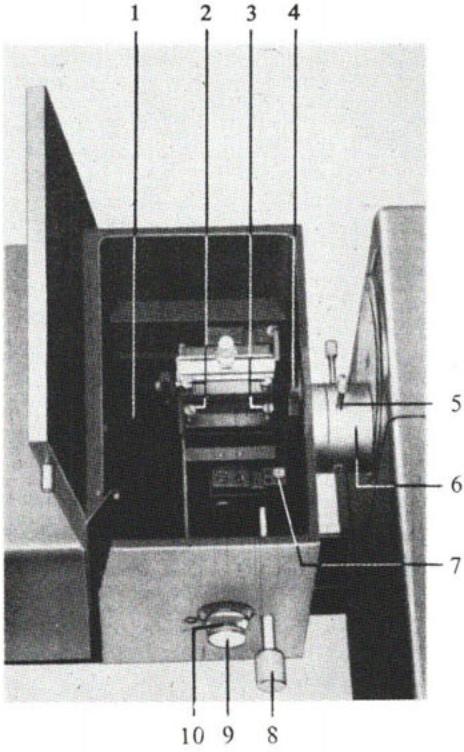

Figure 2. Sample changer for PMQ II with inserted microcuvette

1. short tube for reception of lens
2. and 3. screws for adjusting the height of the cuvette support
4. cross-hole screw for holding and adjusting lens
5. screws for adjusting lens
6. tubular adapter
7. screw for lateral adjustment of the cuvette support
8. pull rod for the cuvette changer
9. knurled screw for limiting the lateral movement of the cuvettes
10. lock nut.

width required for absorbancy measurements with the microcell equipment only needs to be increased by the factor of 1.7, as compared with macrocells and similar amplification. The sample changer can be fitted

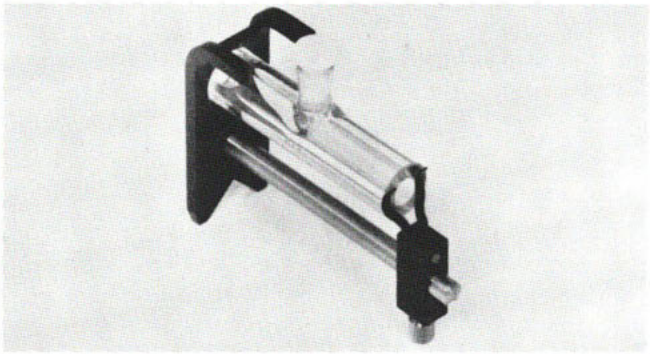

Figure 3a. Tubular microcuvette with holder

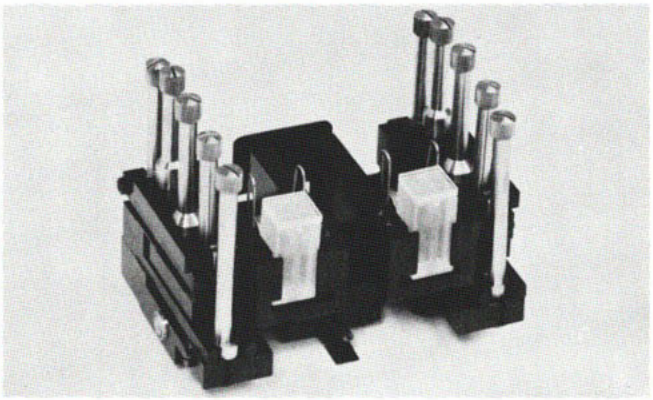

Figure 3b. Trough microcuvette with holder

with holders for trough microcells (Figure 3). Microcells with an inside width of 4 mm and up to 5 cm in length and microcells with 2 mm inside width and up to 1 cm length can be used with the optical system if the monochromator slit is not opened more than 0.8 mm. Precise adjustment of the microliter cells is easy to achieve by a built-in magnetic device. With careful adjustment the amount of sample required is 200 μl/cm light path for the 4-mm and 100 μl/cm light path for the 2-mm microliter cuvette.

In the clinical laboratory, trough-type microliter cuvettes are preferred, mainly because optical enzyme tests, which require mixing of the assay medium in the cuvette, can be performed more easily than in tubular microcells. The standard cuvette changer (without optical system) can be fitted with a movable slit diaphragm having four different slit widths, and a magnetic positioner for independent adjustment of four trough microliter cuvettes.

Beckman DU spectrophotometer

An adapter for microanalysis with the Beckman DU spectrophotometer was first described by O. H. Lowry in 1946. Beckman Instruments now furnishes a microcuvette system for models DU and DU 2. The system consists of a circular micro-aperture plate with eight pinholes of various sizes, a microcell holder with devices for individual vertical adjustment of four microcuvettes, and a microcell sample carrier. Six sizes of microliter trough cuvettes with 10-mm light path are available for measurements with volumes ranging from 50 to 1200 μl.

Eppendorf Photometer

The Eppendorf photometer (Figure 4) is equipped with a series of monochromatic glass and interference filters for the isolation of the commonly used spectral lines between 313 and 1014 nm. The radiation source is a mercury vapor or combination mercury-cadmium vapor lamp. Three photocells of different spectral sensitivity are available for model 1100; model M 1100 is equipped with a photomultiplier.

Figure 4. Eppendorf Photometer with recording accessory.
(Netheler & Hinz, Hamburg, West Germany, Brinkmann Instruments, USA).

The cuvette holder receives three cuvettes. A slit diaphragm is supplied for use with microcuvettes of 2-mm width and a light path of 10, 20 and 40 mm. With careful vertical adjustment the minimum volume required in microcells is 110 μl per 10 mm length. The Eppendorf photometer employs direct reading of absorbance with partial compensation by a set of calibrated resistances. The ready accessibility of the cuvettes permits quick operation with this precise, stable and sturdy instrument.

Gilford spectrophotometers

Trough-type microliter cuvettes with 10-mm light paths can be used with the various Gilford spectrophotometer models. Pinhole diaphragms and a special cell holder for independent vertical adjustment of four microliter cells permit the reduction of the working volume to about 40 μl.

The Gilford 300 and 300-N microsample spectrophotometer is designed for speedy operation, and it features an automatic sampling system which permits about five samples to be read per minute. The 10-mm microcuvette holds a volume of only 120 μl, but at least 500 μl sample are needed for filling. This fact unfortunately excludes this instrument for use in the majority of the microliter methods described in this book, most of which have a final assay volume between 200 and 300 μl.

A model 300-N was tested in our laboratory and the performance was as described by the manufacturer. It would seem possible that the manufacturer could redesign the sampling system in order to eliminate some of the dead space and to reduce the required filling volume to 200–250 μl.

Spectronic 20 (Bausch and Lomb)

The Spectronic 20 is equipped with a diffraction grating system and a tungsten lamp as radiation source. The wavelength can be set between 340 and 650 nm, and the range can be extended by use of a filter and a different photo-tube. A special cuvette holder receives a 100-μl cuvette with 10-mm light path.

The instrument tested in the author's laboratory had a slightly unstable zero point. It is therefore suggested that a second cuvette holder with a cuvette filled with water or reagent blank to permit frequent checking and readjusting of the zero point be available. The scale, absorbance, and % transmission is small, and exact reading, particularly of the absorbance, is therefore difficult.

Beckman-Spinco 151 Spectro-colorimeter

This instrument is part of the Beckman-Spinco Ultramicro Analytical System (page 29) designed by Sanz. A glass wedge interference filter covers the wavelength between 400 and 600 nm. The small scale (absorbance and %-transmission) does not permit exact reading. The 100-μl cuvette is stationary and connected to a suction pump for evacuation. The light path of only 6.4 mm is a decisive disadvantage and leads to low and inaccurate reading with many color reactions.

The instrument tested in our laboratory was unstable, and the reproducibility of various microanalyses was unsatisfactory. Reports from other authors are more favorable, however, and we must mention that we probably had a poorly functioning instrument at our disposal.

The basic idea of Sanz for a microphotometer is certainly a good one. One can only hope that the manufacturer revises the construction of this instrument, particularly because other parts of the analytical system are very useful.

SPECIAL DEVICES FOR PHOTOMETRY

Automated photometers

Automatic cuvette changers are available for several photometers. They permit continuous and simultaneous recording of absorbance changes in four to six cuvettes.

Self-emptying microcuvettes

Self-emptying microcuvettes operated in connection with a suction pump are available for some of the photometers. The carry-over from the previous sample normally does not exceed 1% of the volume, and rinsing between samples is unnecessary unless samples with great absorbance differences are measured in succession. In this case prerinsing with about 50 μl of the following sample is suggested. In serial analyses in our laboratory we prefer to empty the cuvettes by suction from the top. The cuvettes are left in the cell holder and the fluid is removed with polyethylene tubing attached to a strong vacuum pump. After the fluid is removed, the cuvette is flushed once with water: by moving the tubing up and down and back and forth several times, the water can be completely removed.

An automatic filling and emptying device is available for the Spectronic 20, but it is not suited for microliter analysis. Although the cuvette holds only 100 μl, 1.5 ml of sample are needed for flushing and filling.

Zeiss microcuvette MR 1 D

This special cuvette holds 20 μl and is designed as a flow-through cell (Figure 5), but a simple device can be made in the laboratory to measure single samples: one of the outlets is fitted with a 2–3-cm long piece of polyethylene tubing. With the technique described on page 17

(constriction pipets), press in a constriction a few millimeters above the outlet, using a pair of pliers shown in Figure 10. To the other outlet fit a longer piece of tubing and attach to it a three-way stopcock. Connect one outlet of the cock to a suction pump, the other to a Hamilton microliter syringe. To fill the cuvette, use a polyethylene pipet with a long tip to transfer between 30 and 35 μl of sample into the tubing with the constriction. With the cuvette in the light beam, draw the fluid slowly

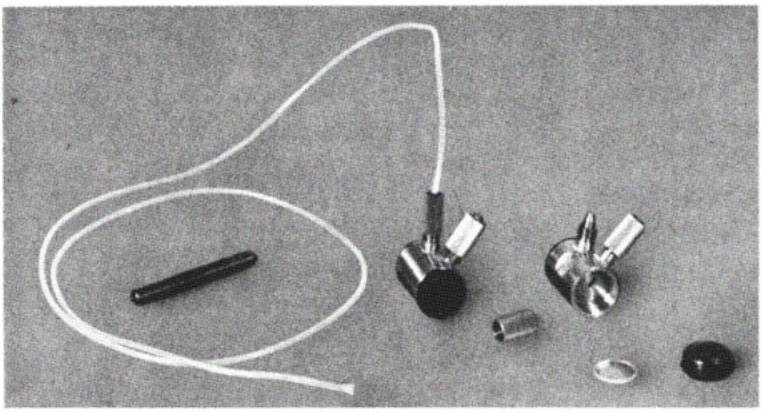

Figure 5. Zeiss microcuvette MR 1D. Volume 20 μl. Designed as a flow-through cuvette; use for single measurements is possible (see text).

into the cuvette with the syringe, and watch the indicator scale. As long as the cuvette is not completely filled or air bubbles trapped, the absorbance will be extremely high. By carefully moving the plunger back and forth, one will find an absorbance minimum when the cuvette is filled completely and bubble-free. The cuvette is flushed and rinsed by suction.

This technique is of course not recommended for routine measurements, but it can be used for special purposes when very little sample is available.

Temperature-controlled cell holders

Enzyme determinations, with the optical test, performed directly in the cuvette, require a temperature-controlled cell holder. These holders or thermospacers are available for most instruments. A special holder is needed for the short microliter cuvette, which we prefer in our laboratory. The temperature-controlled cuvette holder furnished with the Eppendorf