ULTRAMICRO METHODS

FOR CLINICAL LABORATORIES

KNIGHTS · MacDONALD · PLOOMPUU

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Edwin M. Knights, Jr., M.D.
Associate Pathologist, Director
of Clinical Pathology and Blood Bank

Roderick P. MacDonald, Ph.D.
Director of Clinical Chemistry
and Research Advisor

Jaan Ploompuu
Chief, Division of Ultramicro Chemistry
and Research Associate

All of Harper Hospital, Detroit

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Foreword

The employment of accurate and rapid ultramicro methods for quantitative determination of chemical constituents of blood or other body fluid represents an important advance in the clinical laboratory.

The need for such methods is readily recognized whenever only minute amounts of material are available, as in infants, children and small experimental animals; whenever superficial veins are not accessible, available, or adequate for withdrawal of blood; or when frequent sampling or multiple testing of blood is desired, even in adults with large and easily accessible superficial veins.

Until now these methods have been employed mainly in larger clinical laboratories or in biologic experimental studies, inasmuch as an extra measure of skill and care is necessary. Compared with the grosser methods the performance of ultramicro technics requires less space and permits economy of equipment and reagents. Clinically their employment in selected patients has been advantageous to the patients and has regularly proved popular with the attending physicians. Ultramicro chemical methods are now a valuable adjunct to modern clinical pathology.

In presenting this manual on "Ultramicro Methods for Clinical Laboratories," the authors have rendered a distinct service to clinical pathology, making available in ready form a number of practical technics. In view of the constant development of new and refined methods in this field one can confidently expect frequent and repeated revisions and additions in subsequent editions of this work.

S. E. Gould, M.D.
Professor of Pathology
Wayne University
College of Medicine

Preface

The increased use of hospital laboratories in diagnosis of human disease has been little short of phenomenal. Today's medical school graduates, trained in the value of maintaining proper electrolyte balance and familiar with recent innovations in clinical pathology, have placed an unprecedented demand on laboratory facilities. Meanwhile hospital laboratories have made continual progress in devising and improving methods to supply the clinician with accurate blood chemistry results. The history of clinical biochemistry has been one of continual progress with modification superimposed upon modification.

All of this interest in blood chemistry has resulted in greater amounts of blood being removed from the sick patient. This has caused considerable concern to many who feel the leech has been abandoned only to be replaced by the medical technologist. There should be some method to improve the situation other than by purchasing larger sized syringes. The problem becomes particularly acute in those cases involving immature infants, extremely obese patients, third-degree burn cases, or patients requiring intravenous feeding. Here it would be particularly desirable to obtain blood by a method other than venipuncture, since it is often necessary to perform determinations on relatively small amounts of blood.

The analysis of blood samples smaller than those usually available for routine technics has usually been approached by attempts to use "cut down" versions of macromethods of analysis. The disadvantages of this approach, whether by titrimetric, colorimetric or gasometric methods are quite apparent. The apparatus and procedures of the tests have been designed for larger aliquots; reducing the sample size often tends to introduce considerable chance of error. Usually the original examination has been prepared so as to give a reading in the optimal titrimetric or spectrophotometric range and deviation from this range may greatly decrease the sensitivity and accuracy. Often the amount of blood required still necessitates a venipuncture. An alternative approach to this problem is the use of ultramicro methods of analysis. The exact definition of *ultramicro* is difficult to explain but is generally considered to include determinations of micrograms (0.001 mg.) or microliters (0.001 ml.). As the individual test requires only 0.01 to 0.03 ml. of serum, a fairly complete survey can be performed on 0.2 to 0.3 ml. of blood obtained by pricking the heel or finger. The methods are also particularly well adapted to the examination of small quantities of cerebrospinal fluid.

Microgram analytical methods have already made significant contributions to such diversified fields as enzyme histochemistry, tissue cultures, and protozoology but it is only recently that very extensive clinical use has been made of them. Such investigators as Rappaport, Glick, Natelson, Kirk, and Caraway have done much to further our knowledge of their clinical usage. Gradually more and more hospitals are adopting ultramicro methods as a solution to their problems, and as interest in the technic grows the need for a practical manual of methods for hospital use becomes more acute. It is sincerely hoped that this book will fill that need. As Otto Schales said in his presidential address to the Seventh Annual Meeting of the American Association of Clinical Chemists, "All of us have encountered the dilemma of a battery of tests to be done with insufficient blood. Ultramicro methods will be of great value in such instances."

Unfortunately inadequate knowledge of ultramicro methods combined with excessive demands on already existing facilities and personnel in hospital chemistry departments has resulted in their rather slow acceptance. Actually, once the ultramicro division has been established it proves itself remarkably adaptable to routine use and results can be obtained with an accuracy that compares favorably with standard procedure. But in order to achieve such accuracy samples should be obtained and analyzed by competent technologists trained in this subspecialty. As the equipment, reagents and glassware are used only by the ultramicro chemist, they should be his personal responsibility and kept exclusively for his use. Ultramicro chemistry has a distinct advantage in the minimum amount of space required for its operation; however, it is very desirable that it be kept as separate as possible from the frenzied activity of the routine hospital chemistry department. Once in operation it proves extremely valuable, not only in the analysis of blood specimens from the previously mentioned types of patients but also in providing a convenient means of checking questionable results obtained with macromethods where only a small amount of serum is still available. The potentialities of the ultramicro method in research have barely been explored. When combined with the use of microhematocrit methods it is extremely useful in the management of difficult pediatric cases. It can also be expanded to include the fields of paper chromatography and paper electrophoresis. Such laboratory tests as non-protein nitrogen, urea nitrogen, total protein, creatinine, albumin, glucose, sodium, potassium, chloride, icteric index, bilirubin, cholesterol, thymol turbidity, cephalin flocculation, phosphatase, amylase and carbon dioxide combining power, have proved adaptable to ultramicro methods and provide a well-rounded selection for the practicing physician.

THE AUTHORS

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Setting Up Ultramicro Chemistry in the General Laboratory

In the general hospital laboratory an ultramicro division may be established as a subdivision of the chemistry department, but it is best to keep it quite separate physically from routine chemical procedures. At Harper Hospital ultramicro chemical methods and research had been used for many years, but attempts to incorporate them into the hospital routine were unsuccessful until a separate subdivision had been properly organized and equipped. As the ultramicro chemist must work as quietly and with as little disturbance as possible he should be separated from the routine macro chemical procedures. A sink with suction attachment and a handy reagent shelf are important. In our laboratory a three-decked "lazy Susan" type of dispenser puts all of the necessary reagents at the technologist's fingertips without necessitating his leaving his seat.

Since the equipment, reagents, and glassware are used only by the ultramicro technologist they should be kept exclusively for his or her use. A possible exception to this is the flame photometer on which the sodium and potassium determinations are performed. The technologist must be completely responsible for the glassware and its maintenance. Results can be obtained with an accuracy comparable to standard procedures, but in order to achieve this samples should be obtained and analyzed by competent technologists who have received additional instructions in this subspecialty.

A small group of selected reference books and pertinent articles will always be of considerable value for consultation and these should be located conveniently in the laboratory for the use of the ultramicro technologist. We have included here a list of reference works that form a small "library" of useful and important information.

CARAWAY, W. T., AND FANGER, H.: Ultramicro procedures in clinical chemistry. *Am. J. Clin. Path. 25*: 317-331, 1955.

KAPLAN, S. A., AND DEL CARMEN, F. T.: Quantitative ultramicro-analysis for the clinical laboratory. *Pediatrics 17*: 857-869, 1956.

KING, E. J.: *Micro-Analysis in Medical Biochemistry*. London: J. & A. Churchill, Ltd., 1951.

KIRK, P. L.: *Quantitative Ultramicroanalysis*. New York: Wiley & Sons, 1950.

MILTON, R. F., AND WATERS, W. A.: *Methods of Quantitative Microanalysis*. London: Edward Arnold & Co., 1949.

NATELSON, S.: Routine use of ultramicromethods in the clinical laboratory. *Am. J. Clin. Path. 21*: 1153-1172, 1951.

NATELSON, S., CRAWFORD, W. L., AND MUNSEY, F. A.: *Correlation of Clinical and Chemical Observations in the Immature Infant*. New York: Endo Products, Inc., Richmond Hill, 1952.

RAPPAPORT, F.: *Rapid Microchemical Methods for Blood and CSF Examinations*. New York: Grune & Stratton, 1949.

SOBEL, A. E. AND HANOK, A.: Ultramicro quantitative analysis in clinical laboratories. *Mikrochemie 39*: 51-68, 1952.

STRUMIA, M., SAMPLE, A. B., AND HART, E. D.: An improved microhematocrit method. *Am. J. Clin. Path. 24*: 1016-1024, 1954.

Before ultramicro procedures are adopted by a laboratory, careful checks are necessary on each of the procedures. Three types of controls are necessary:

1. The methods should be run on venous blood in comparison with macromethods on the same sample.

2. The ultramicro methods on capillary blood should be compared with macro procedures performed on venous blood which has been obtained from the same patient at the same time.

3. Controls consisting of samples of known value should be run concomitantly.

A study by Belk and Sunderman in 1947[1] led many laboratory directors to consider the necessity of strict control of chemical methods. Levey and Jennings first proposed a control chart, using pooled specimens of serum or plasma as standards. This technic has been developed by others[2, 3] to the point where it offers a practical and simple method of determining laboratory precision and reproducibility.

A batch of pooled human serum is prepared by adding sufficient glucose and bilirubin to bring the levels of these constituents up to 100 mg. per cent and 2 mg. per cent respectively.[2] For ultramicro use this serum is divided into individual samples of about 0.5 ml. each and placed in a deep-freeze until required. The individual specimen is thawed at about 30-35 C. on the day of use. At least 30 determinations on individual days are required

to begin the statistical analysis. The standard deviation for each method is calculated from these data as follows:

$$\sigma = \sqrt{\frac{\Sigma\ (\bar{x} - x)^2}{N - 1}}$$

σ = *standard deviation*
Σ = *"sum of"*
$\bar{x}$ = *arithmetic mean of* N *determinations*
x = *a single determination*
$\bar{x} - x$ = *deviation of a single determination from the mean*

The term "standard deviation" is a measure of the dispersion of a group of values about their mean. To make standard deviation useful "allowable error" is calculated from it and used for subsequent determinations.

$$\text{Allowable error (in per cent)} = \frac{3\sigma \times 100}{M}$$

σ = standard deviation
M = mean

A control chart is prepared for each determination with the analytical values (expressed in mg. per cent, mEq./L., etc.) as the ordinate and the data the test was performed with as the abscissa. The mean obtained from the statistical study is drawn as a line parallel to the x-axis and parallel lines drawn to show the limits of allowable error.[3] Comparison of any subsequently determined value with the values on this chart will show whether or not the method is "in control."

Benenson, et al[2] list as the common factors causing a method to go out of control: deteriorated or faulty reagents, variable laboratory conditions, careless technic, and poorly trained or inexperienced personnel. An active program of laboratory control will serve to detect any of these situations.

Reference standard solutions may be analyzed concurrently with any unknown specimen and in some ultramicro methods this technic is suggested as a daily routine. It will be especially helpful in the determination of an end-point with which the technician is not familiar. Commercially prepared products are:

1. Lab-Trol (Dade Reagents, Inc.) which contains known amounts of protein, glucose, urea nitrogen, chlorides, phosphorus, calcium, sodium and potassium.

2. Versatol (Warner-Chilcott Laboratories) which contains known amounts of protein, sodium, potassium, glucose, urea nitrogen, phosphates, calcium, and glucose.

3. Clinical Pathology Standards (College of American Pathologists). Separate ampules containing known amounts of glucose, nitrogen, chloride, calcium, uric acid, creatinine, phosphate, sodium, and potassium.

These and other products mentioned throughout the book may be obtained from the firms listed. The addresses of these suppliers have been placed conveniently in the Appendix, page 126.

Calculations in this book refer in many instances to the use of a "Factor." This is derived from the equation:

$$\frac{OD_{unk}}{OD_{std}} \times Conc._{std.} = Conc._{unk.} \qquad (1)$$

$$OD_{unk} \times \frac{Conc._{std.}}{OD._{std.}} = Conc._{unk.} \qquad (2)$$

a b

Expression b in the second equation is constant for a given series of analyses and is referred to as the "Factor." The final calculation is thereby reduced to a single step. In the calibration procedure this factor is determined for a range of levels and the average is used *unless* there is a marked deviation at either high or low concentrations. In this case a calibration chart should be used. The use of a factor is valid only when the color produced conforms to Beer's Law and consequently this relationship must be predetermined in the calibration procedure. Standards and control specimens should be used periodically to validate the accuracy of the method.

REFERENCES

1. Belk, W. P. and Sunderman, F. W.: *Am. J. Clin. Path.* 17: 853, 1947.

2. Benenson, A. S., Thompson, H. L. and Klugerman, M. R.: *Am. J. Clin. Path. 25*: 575, 1955.

3. Henry, R. J. and Segalove, M.: *J. Clin. Path. 5*: 305, 1952.

4. Levey, S. and Jennings, E. R.: *Am. J. Clin. Path. 20*: 1059, 1950.

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Special Equipment

Microgasometer

The Kopp-Natelson microgasometer (Model 600) is used. It is a manometric instrument based on the classic Van Slyke method. The mercury reservoir is controlled by a hand wheel and gas pressure is measured under constant volume so that results are not dependent on atmospheric pressure. Serum samples of only 0.03 ml. are required. Complete instructions for the use of this instrument are provided by the manufacturer (Scientific Industries, Inc.) and a microburet attachment is available (FIG. 1).

Ultramicro Buret

A specially designed ultramicro buret should be used, preferably one with 1.0 ml. capacity in 0.001 ml. divisions. The Gilmont combination micro pipet-buret (G 15395A, Emil Greiner Co.) is a satisfactory instrument. It has a synthetic ruby plunger ground to the proper diameter so that the dial gauge reads directly in volume units. This displaces the fluid from a reservoir sealed by a polytetrafluoroethylene gasket.[1] The Schwarz-Rehberg or Kopp burets may also be used, but the Gilmont modification of the Scholander model does not require the use of mercury and is relatively easy to operate (FIG. 2). The Kopp-Natelson microgasometer may be fitted with a specially designed adapter to permit its use as a microburet. A very simple ultramicro buret may be constructed in the laboratory from a pipet, rubber bulb and screw clamp according to Hepler[39] or more complex models may be made using tuberculin syringes and a differential screw mechanism.[40]

Pipets

Constriction type ultramicro pipets in 10, 20, 25, 50, 100, 200, and 250 lambda sizes are most satisfactory for clinical laboratory use, and they are calibrated to deliver. It is simplest to purchase these from commercial sources (Microchemical Specialties Co.) but they can be manufactured and calibrated in the laboratory as described by Natelson.[13] See the next chapter, *Collection of Blood and Pipetting*, for additional details.

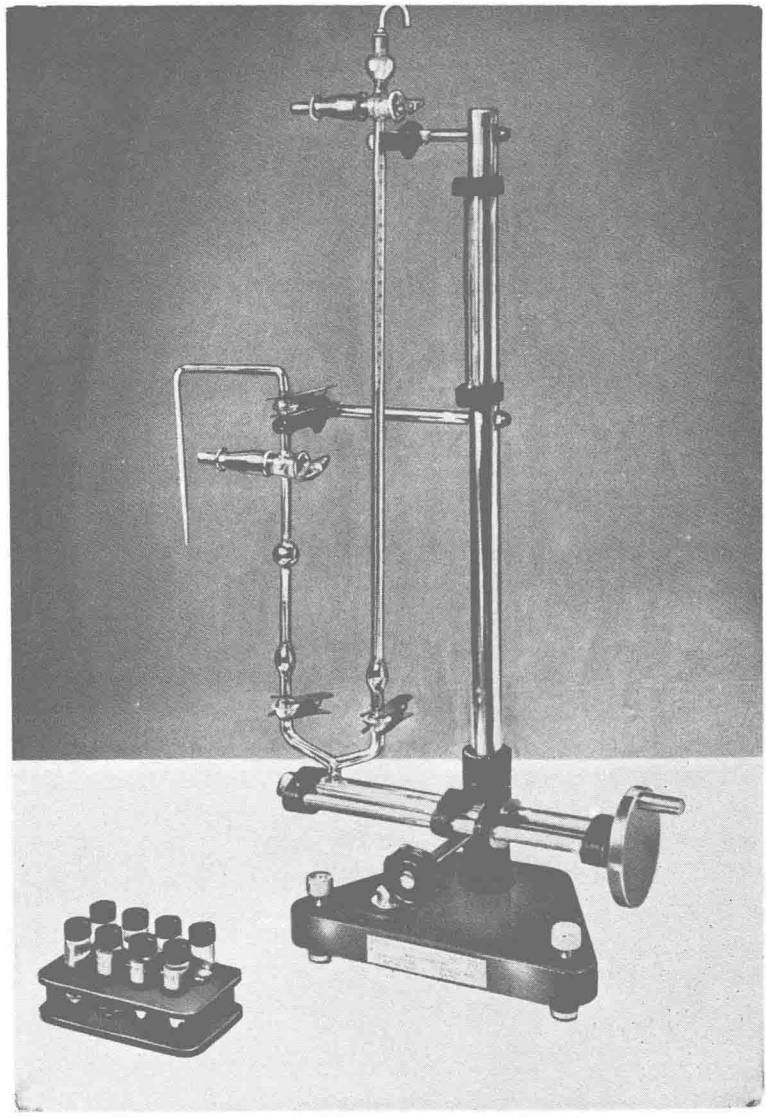

Fig. 1.—Kopp-Natelson microgasometer, Model 600.

Centrifuges

Several types of centrifuges are used:

1. A standard model of any reliable make is satisfactory for centrifuga-

tion of clotted blood collected in capillary tubes since the capped or sealed tubes may be placed within macro-sized tubes for centrifugation.

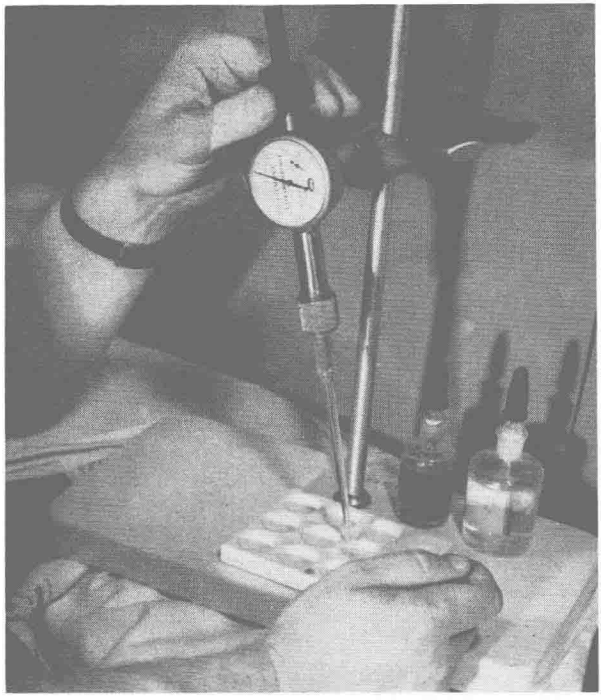

Fig. 2.—Gilmont combination micropipet-buret.

2. For separation in the 1.0 ml. ultramicro centrifuge tubes, a Misco electric micro centrifuge may be used. This is a sturdily built model modified from Kirk's air turbine ultramicro centrifuge [14] and provides speeds up to 22,000 r.p.m. controllable by a separate rheostat (FIG. 3).

3. If it is desired to perform microhematocrits in conjunction with the ultramicro chemistry, specially designed high speed capillary centrifuges may be purchased.[15-16] Two of these are the Hemacrit (International Equipment Co.) and the Drummond microhematocrit centrifuge, clinical model. (Drummond Scientific Co.)

Beckman DU Spectrophotometer

The Beckman DU spectrophotometer (FIG. 4) has been modified for ultramicro analysis[17-18] and is particularly suitable because of its narrow

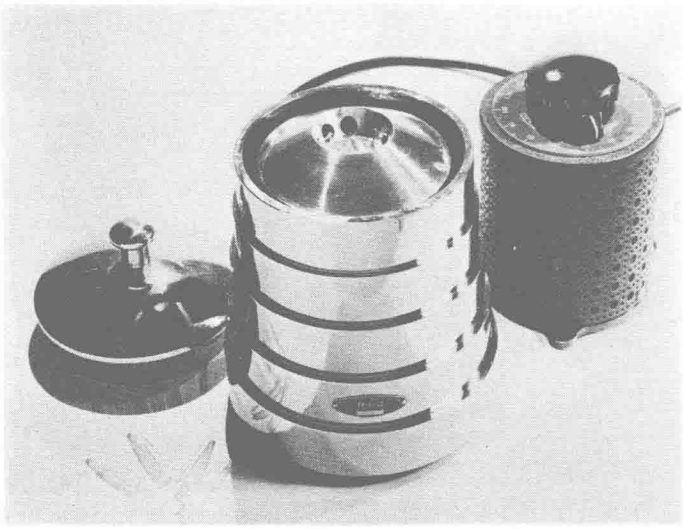

Fig. 3.—Misco ultramicro-centrifuge with rheostat.

beam of monochromatic light and sensitive photometer for light detection. For the clinical laboratory the Bessey Lowry cells recommended by Caraway[19] are easy to fill and adjust. (Pyrocell Manufacturing Co.) These cells are of fused silica, which is highly transparent over the entire spectral

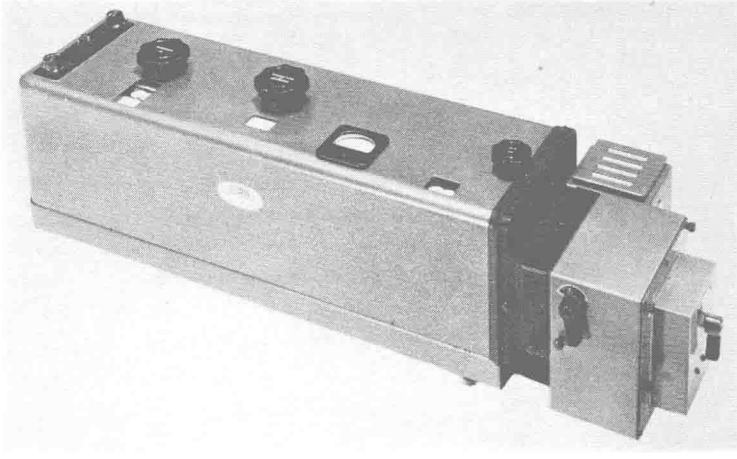

Fig. 4.—Beckman DU spectrophotometer.