Minipette system, a convenient ultramicro performance of routine clinical chemistry by means of a semi-automatic pipet (minipette) and a drain-system colorimeter of new design.

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Two major directions may be distinguished in the trend of clinical chemistry today. The first one is automation system represented by the use of autoanalyzer and robot chemist, trying to complete all the quantitative determinations automatically with instruments without using human hands.[1] The second one is an ultramicro determination with the smallest possible amount of samples.[2~5] We have dedicated our efforts of several years along the second direction (ultramicro determination) and successfully developed an original system which enables simultaneous and semiautomatic chemical analysis of 14 to 15 components with only 0.5 ml of serum. The outline of this system will be briefly described.

From the standpoint of clinical chemistry the scale of analysis is defined as follows : —The methods are classified according to the amount of the sample used for analysis. When the volume of the sample exceeds 1.0 ml the method is called macroanalysis. Microanalysis deals with samples between 0.1 and 1.0 ml in volume. Samples less than 0.1 ml must be analyzed by an ultramicro analysis. Ultramicro analysis is therefore said to be the quantitative chemical analysis, using less than 0.1 ml of serum as sample.

Necessity of ultramicro anlysis has become apparent from the fact that the amount of blood sample collected from the patient is relatively small in spite of many kinds of determinations ordered to the laboratory from the ward. Difficulty is large, when conventional techniques of microanalysis is used for seriously ill patients and infants. Collection of blood from them is not easy. In addition, simultaneous analyses of more than 10 blood components and subsequent interpretation of data are frequently necessary to establish the clinical diagnosis.

About 10 years ago Professor Shibata[6,7] suggested a system of diagnosis based on clinical chemistry which is called "blood spectrum" to facilitate the diagnosis. The "blood spectrum" requires simultaneous analysis of 10 or more components with a limited amount of blood. So, the amount of sample (serum or blood) for chemical analysis should be kept as small as possible. Ultramicro analysis has accordingly be urgently needed in our laboratory.

Ultramicro analysis has been investigated by many workers especially in the

U. S. A., and at present commercial instruments for this purpose have become obtainable from Beckman-Spinco (USA) Co., and Coleman (USA) Co. and so forth.

Unfortunately, however, these instruments have been designed for taking very small amounts of reagents, necessitating so much attention and care in manipulation that their application to routine clinical chemistry becomes impossible. Accordingly, at the present stage ultramicro analysis is opened only for skilled technologists.

What should we do in order to develop a routine ultramicro analysis to which every ordinary technologist is accessible?. Attempt at simple diminution of the amount of reagents involved in reactions may impose undue nervous tension on technologists without improvement in efficiency. Discovery and application of a new sensitive chemical reaction for ultramicro analysis demands extensive basic training in analytical chemistry which is usually beyond the capacity of clinical chemists of medical career.

Since ordinary micro methods of analyses use 0.1 to 0.2 ml of serum and make the final volume of colored solution for colorimetry to the level of 5–6 ml, we have endeavored to reduce these volumes to approximately one-fifth as small, namely to 0.02 ml (or 20λ) of serum and to 1 ml of colored solution so that the procedures may be conducted easily without paying special attention and skilfulness in handling. In addition. we have made every effort to simplify the procedures to one-step method, if possible, in order to minimize the sources of the intervention of technical errors.

INVENTION AND IMPROVEMENT OF THE INSTRUMENTS[8]

Our effort was concentrated for a long time around the invention and improvement of a convenient colorimeter and an instrument for pipetting small amounts of reagents. They were rewarded with our creation of the two instruments which will be described below: (1) a newly designed drain-system colorimeter and (2) a "minipette" for semiautomatic pipetting.

A) Photoelectric colorimeter[9] (Photograph 1): Commercial ADS photoelectric colorimeter was improved so that rapid colorimetry of colored solution of 1 ml in succession may be feasible. The cuvette is not movable, being fixed to the colorimeter. Its construction is shown in Figure 1. Dead

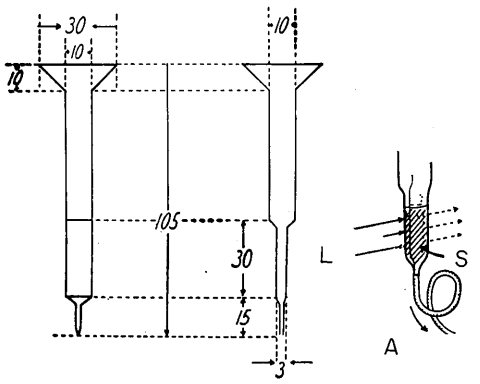


Fig. 1: Fixed cuvette with drain-system in S-ADS photoelectric colorimeter. A: To the aspirator. L: Light. S: Colored solution in the cuvette. (measure: mm)

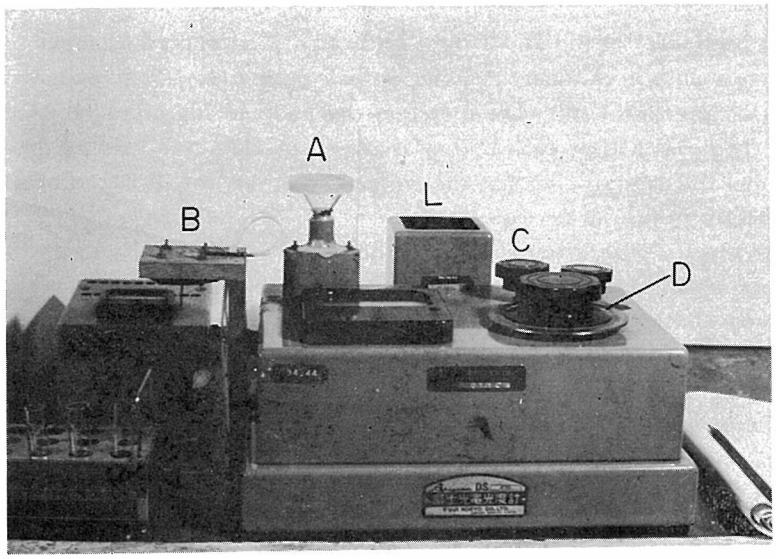


Photo. 1. S-ADS photoelectric colorimeter for ultramicroanalysis.
A : Cuvette. B : Trap for the recovery of colored solution from the cuvette after reading of optical density is over. C : Fine zero adjustor. D : Dial for reading optical density. L : Light source.

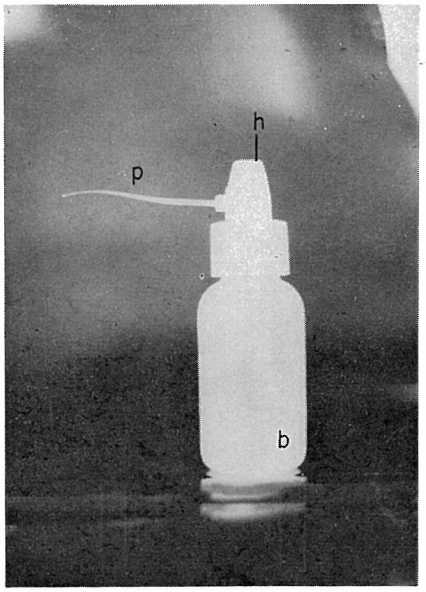


Photo. 2. Sanz's ultramicropipet. b : Polyethlene bottle. h : Hole for the air. p : pipet or beak (20λ).

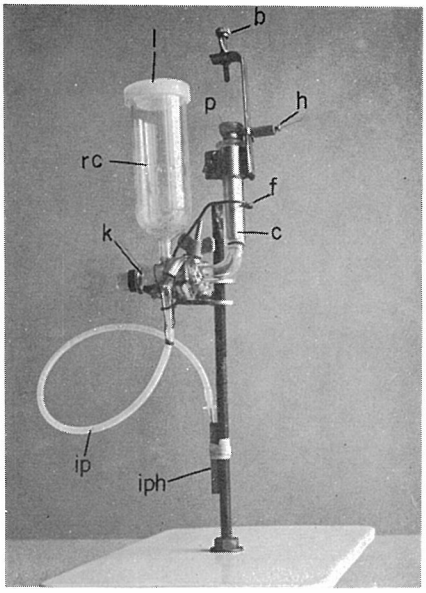


Photo. 3. Minipette. b : Screw-nail for adjusting the amount of reagent to be taken. c : Barrel of the syringe. f : Holding frame for fixing the barrel of the syringe. h : Handle. ip : Tube for introducing the reagent into the receiving test tube. iph : Holder for the introducing tube. k : Coordinated cock. l: Lid for reagent container rc : Reagent container.

space on both sides of the direct light path-way was reduced as much as possible in order to manitain the length of the optical path over 1.0cm with its effective capacity of less than 1 ml. Drain system was adopted for rapid removal of solutions in the cuvette after colorimetry is over. A trap was inserted between the cuvette and the aspirator so that the colored solutions might be recovered in test tubes directly in case of necessity.

Simple touch of the lower surface of the holder of the trap with the mouth of a test tube is sufficient to produce negative pressure which enables us to aspirate the colored solution from the cuvette into the test tube.

B) Sanz's ultramicropipet[10)11)] (Photograph 2): In our experience, this is the best equipment for sampling 0.02 ml (20λ) of serum.

C) Minipette[8)] (Photograph 3): In ultramicro analysis reagents of as small as 0.1 to 0.2 ml may often be measured and delivered in rapid succession. This procedure, namely pipetting or buretting small amount of solution in the bottleneck in the performance of ultramicro analysis, since all the instruments so far available for ultramicro analysis as well as newly proposed ones fail to eliminate the difficulty of manipulation. After continuous efforts of 4 years we have successfuly developed a satisfactory apparatus which is designated "minipette". This instrumet (Figure 2) is composed of a syringe (plunger and barrel), a container for reagent, and a stop-cock coordinated with the operation of the syringe synchronously. When its handle is moved downwards, the plunger goes down because of its own weight preparing a vacant space in the barrel, to which reagent is introduced from the reagent container spontaneously. Thus the barrel is filled with a certain fixed

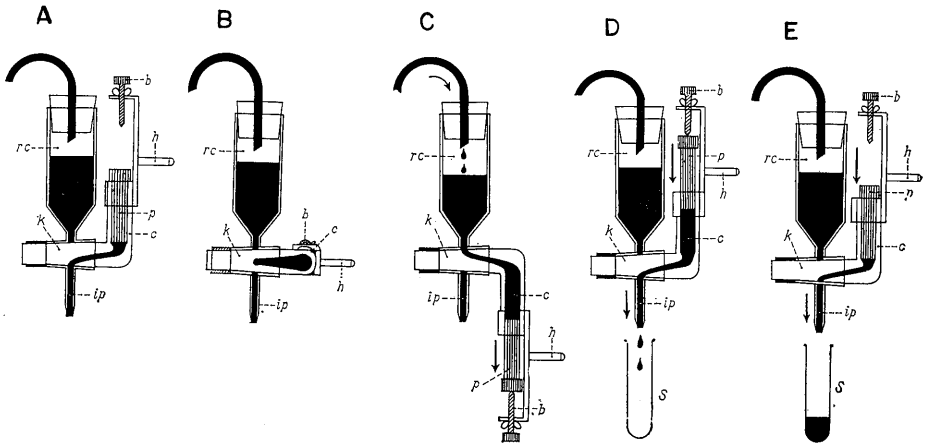


Fig. 2. The principle of minipette system. A: When not in use. B: The handle is moved forwards. C: The reagent is introdduced to the vacant space of the barrel from the reagent container sponaneously when the handle is moved downwards. The reagent container is also supplied with the reagent from the reagent bottle at the same time. D: The handle is ifted. The reagent in the barrel is about to be ejected into the receiving test tube. E: The reagent has now been transferred into the receiving test tube.

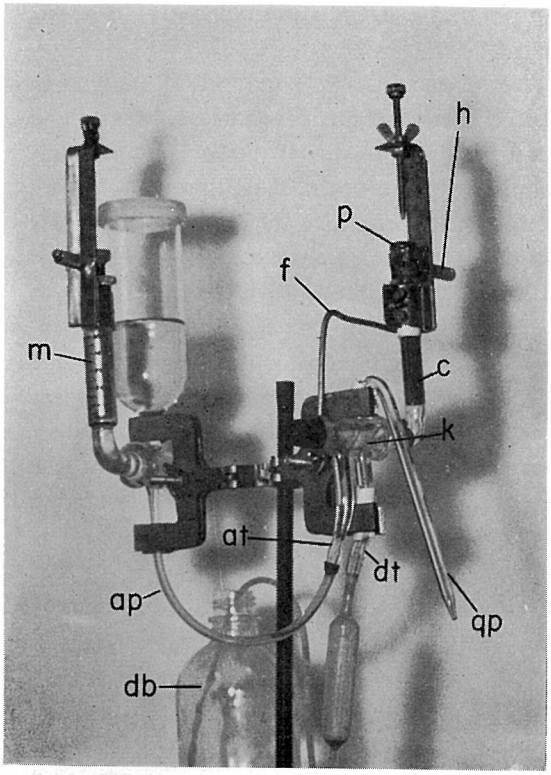


Photo. 4. Seligson-minipette. ap: Tube for aspirating the sample into the pipet (qp). at: Aspiration tube (made of glass). c: Barrel of the syringe for aspirating and ejecting the sample. db: Bottle for receiving wasted reagent and wasted sample. dt: Tube to the bottle of wasted fluids. f: Holding frame for fixing the barrel of the syringe. h: Handle. k: Coordinated cock. m: Minipette. p: plunger of the syringe with its weight at the top.

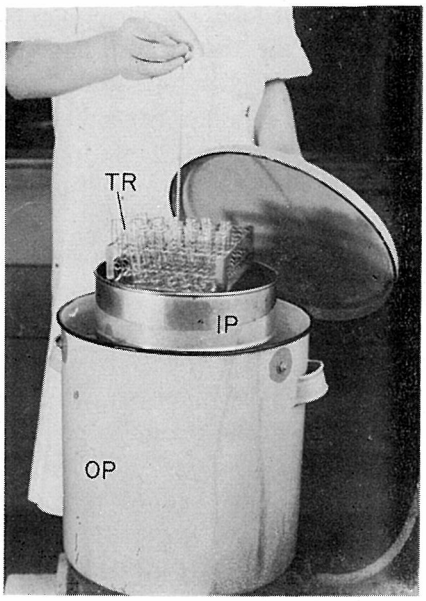


Photo. 5. Double Walled heating bath. G: City gas for heating the outer pot. IP: Inner pot. L: Lid of the inner pot. OP: Outer pot. TR: Test-tube rack.

amount of reagent. Then the handle is lifted. The plunger comes down, pressing the reagent down out of the barrel to make it flow through the stop-cock and the mouth of the instrument into the receiving test tube.

The degree of precision of pipeting 0.1, 1.0 and 3.0 ml of reagent was 1, 0.2 and 0.1 %. respectively, as evaluated from the variation of the weights of 10 samples consecutively pipetted.

D) Diluting apparatus for fractional pipetting[8] (Seligson-minipette), (Photograph 4): This enables us to take with a minipette simultaneously a constant volume of samples and a constant amount of reagent which serves as a diluting solution. Suggestion was obtained from Seligson's pipette.[12] So, it is called Seligson-minipette. Simple upward and downward movements of the handle are

sufficient to introduce and mix the sample and reagent simultaneously in a test tube, with allowable error of less than 1%.

E) Double-walled heating bath[8] (Photograph 5): Several kinds of analyses in clinical chemistry require heating at 100°C (for example, blood sugar, urea N and so forth). It is the daily experience in many laboratories that the reaction mixtures in test tubes are sometimes contaminated with drops of boiling water which spatter in the heating bath. Moreover, the inside of the water-bath is frequently unevenly heated so that some parts are at 100°C whereas in other parts the temperature is significantly lower than 100°C. A double-walled heating bath was therefore designed in order to eliminate these shortcoming. In a stainless steel pot were introdued an adequate amount of water for immersing the bottoms of the test tubes arranged in a rack and a small amount of boiling-point elevating agent such as glycerin. This pot was placed and fixed with nails in the center of a larger pot containing water so that it may be well submerged outside. The lid of the pot was made dome-shaped lest condensed water should fall into the test tubes during heating. The water in the inner pot is heated to 100°C with the heat of the water in the outer pot, which is directly boiled with city gas flame. In this way the water in the inner pot which serves as heating bath is warmed evenly everywhere.

F) Test tubes and test tube racks: Short test tubes like a centrifuge tube with round bottom are preferred to the ordinary test tubes, because the the samples should be pipetted with Sanz's ultramicropipet which has a short beak. The tubes of 75 mm in length is suitable, and three kinds, 10 × 75 mm, 12 × 75 mm, and 15 × 75 mm, are used in our laboratory.

Small test tube racks are therefore prepared for these tubes.

G) Test tube rack designed for CCFT (Photograph 6): This is a test tube rack equipped with a cover-lid for preventing the contents of the small tubes from exposure to light. It is also convenient for keeping even warming in a water bath of constant temperature (37°C).

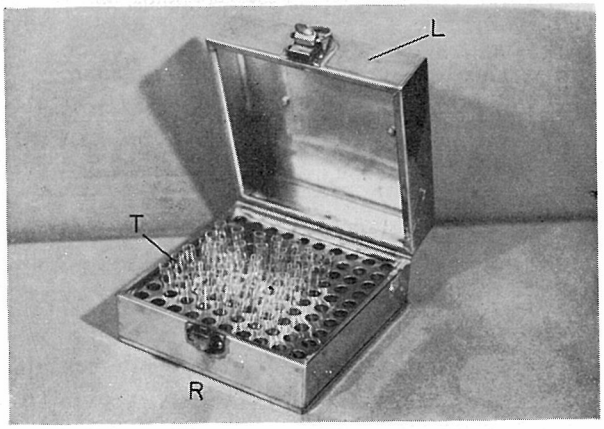


Photo. 6. Test-tube rack for CCFT. L: Lid. R: Rack for test tubes. T: Test tubes.

H) "Minimixer" for mixing and stirring (Photograph 7): It is not so easy to mix or stirr the sample and the reagent in a small test tube

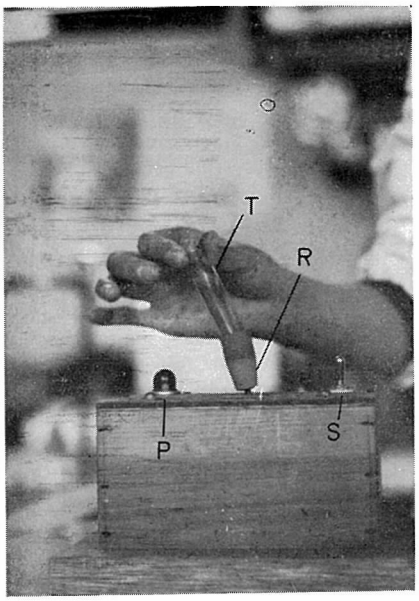


Photo. 7. Minimixer. P: Pilot lamp. R: Rubber cap. S: Switch. T: Test tube

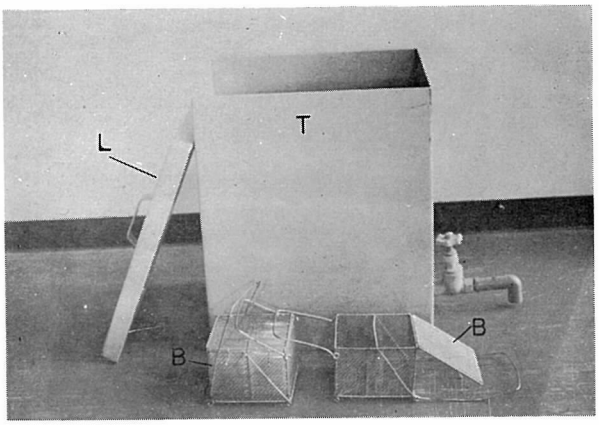


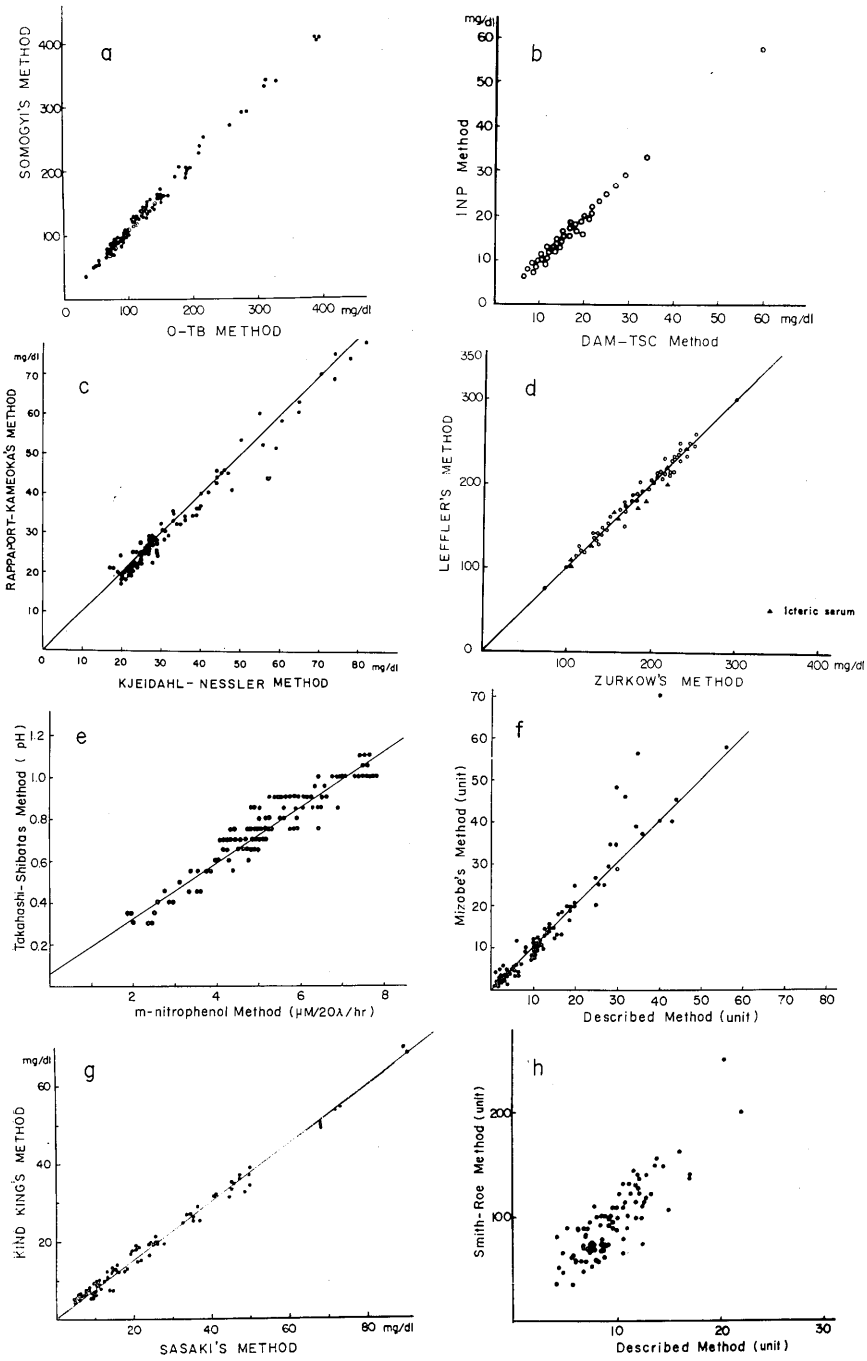
Photo. 8. Washing baskets and a tank containing dichromate-sulfuric acid. B: Washing basket made of stainless steel wire. (Small test tubes are packed in the left basket). L: Lid of the tank. T: Tank made of acid-fast plastic material with a cock for deserting wasted dichromate sulfuric acid solution.

as doing in an ordinary test tube. Although a specially designed apparatus for stirring is commercially available for long test tubes, none has been found for short test tubes. We have therefore constructed a simple stirring apparatus for small test tubes equipped with a motor and a rubber cap.

I) Device for washing test tubes (washing basket, photograph 8): In order to save time and labor for washing thousands of small test tubes individually, a washing basket, which makes washing of 200 test tubes in a mass at one time possible, was constructed. Used test tubes are put in this basket unidirectionally, covered, dipped in potassium dichromate-sulfuric acid solution, taken out of the solution after 2 or 3 hours, and washed in a water tank. The water tank is filled with running water from the tap. The basket is immersed in the water to fill every test tube with fresh water. Then it is taken out and slanted so that the water in the test tubes packed in the basket may be removed completely. Twenty repetions of this procedure is satisfactory to get perfectly washed test tubes.

METHODS FOR THE ANALYSIS

Various conventional methods of analysis were examined to choose the best one for ultramicoanalysis. Every effort was made to rerduce the number of steps and the amount of serum sample as small as possible. The selected methods are presented in Table I. They are all feasible with 20 λ aliquot of samples. Their outline will be described below.


a
SOMOGYI'S METHOD
O-TB METHOD
b
INP Method
DAM-TSC Method
c
RAPPAPORT-KAMEOKA'S METHOD
KJEIDAHL-NESSLER METHOD
d
LEFFLER'S METHOD
ZURKOW'S METHOD
Icteric serum
e
Takahashi-Shibata's Method (pH)
m-nitrophenol Method (μM/20λ/hr)
f
Mizobe's Method (unit)
Described Method (unit)
g
KIND KING'S METHOD
SASAKI'S METHOD
h
Smith-Roe Method (unit)
Described Method (unit)

GLUCOSE[13]

O-toluidine[14] dissolved in glacial acetic acid with minute adequate amount of water and boric acid yields intense blue color when it is mixed with serum or plasma and heated at 100°C for a short time (8 minutes). The optical density of the colored solution is measured at 660 mμ. This is a one-step procedure which is not interfered with bilirubin. Deproteinization is not necessary. The estimations compare quite well with Somogyi-Nelson's micro method[15]. (Figure 3-a).

UREA NITROGEN

Diacetyl monoxime method[16] has been used conventionally for a long time. Its shortcoming is relatively rapid fading of the colored solution. Fortunately, it was found that addition of thiosemicarbazide stabilized the color[17], and presence of protein did not interfere with the color development[18]. One-step procedure was therefore deviced, circumventing deproteinization. The urea nitrogen values obtained by this direct colorimetric method (at 540 mμ) were in good agreement with those measured by the conventional methods.[16] [19] (Figure 3-b)

NON PROTEIN NITROGEN[20]

The principle of hypobromite method[21] was adopted. Hypobromite left unconsumed after it has been allowed to react with NPN was estimated by its capacity of decolorizing phenosafranine in a photoelectric colorimeter (at 540 mμ). The more NPN is present in the sample which was deprotenized by modified Folin-Wu's method[21], the less is the fading of phenosafranine, resulting in higher optical density of the reaction mixture. Colorimerry of phenosafranine constitutes therefore the end point of this NPN determination. This method showed an excellent correlation with the conventional Kjeldahl-Nessler's method[15], although it gave a slightly lower (by 2–3 mg/dl) values. (Figure 3-c).

BILIRUBIN

i) Total bilirubin: Based on the principle proposed by Malloy-Evelyn[22] methanol was added to serum (20λ) to convert hemobilirubin into cholebilirubin.

Fig. 3. Correlationship of the ultramicro procedures to the conventional micromethods. a: o-Toluidine-boric acid (o-TB) method to Somogyi-Nelson's method for glucose, b: Modified Coulombe-Favreau's (DAM-TSC) method to isonitrosopropiophenone (INP) method for urea N. c: Hypobromite-phenosafranine (Kameoka's) method to Kjeldahl-digestion-direct nesslerization method for NPN. d: Modified Zurkowski's (Zurkow's) method to Leffler's method for cholesterol. e: Sasaki's m-nitrophenol method to Takahashi-Shibata's method for serum cholinesterase. f: Sasaki's ultramicro (Described) metod to Mizobe's method for transaminase (GPT). g: Sasaki's untramicromethod to Kind-King's method for alkaline phosphatase. h: Modified Ujihira's (Described) method to Smith-Roe's method for amylase.

This cholebilirubin converted from hemobilirubin was together with the pre-existing cholebilirubin colorized with the conventional diazo reagent. The colored solution thus prepared which was purple in color was measured for its optical density at 540 mμ.

ii) Fractional determination[23 24]: To serum (50λ) was added 1.0 ml of diazo reagent, mixed and allowed to stand at 25°C for 5 minutes. Optical density of thus colored solution was measured in a photoelectric colorimeter (at 540 mμ). The reading (divided by 2) is recorded for cholebilirubin.

The colored solution was recovered from the cuvette into a small test tube completely. To this was added 1.0 ml of methanol and the mixture was measured for its optical density in the same way as before (at 540 mμ). This reading is recorded for total bilirubin.

In case of severely jaundiced serum, blank tube which is prepared with serum (50λ) and blank reagent is necessary.

HEMOGLOBIN

Cyanmethemoglobin method[25] was used without modification of the original procedure.

PROTEIN

Refractometry[26] of serum has been commonly used in many laboratories in this country. This is not so convenient for the disposal of many samples at a time on account of easy fatiguability in recognizing the demarcation line referring to protein concentration. Biuret colorimetric method[27] with 20 λ of serum as sample was therefore preferred by us.

ALBUMIN

Chen's method[28] was modified to ultramicro scale (20 λ of serum). Metachromasia (red) of 4'-hydroxyazobenzene-carboxylic acid-(2) (which is yellow originally) produced in the presence of albumin was employed as the principal reaction for the estimation of serum albumin. The colored solution was measured for its optical density at 480 mμ.

CHOLESTEROL

Zurkowski's method[29] in which cholestrol is stably colorized blue with a modified Liebermann-Burchard's reagent containing sulfosalicylic acid as extra ingredient was miniatured to ultramicro scale. The standard solution was prepared with control serum (Versatol) whose cholesterol concentration was already known to us. The sample and the standard were colorized under the same condition. This ultra-

micromethod compared well with Leffler's micro method[30] which was in use as a routine procedure in our laboratory. (Figure 3-d).

CCFT

In Hanger's original method[31] mixture of serum with CCF antigen solution is incubated at 20°C for 24 to 48 hours to observe flocculation. Martinek et al[32] reported a modified method which enabled the evaluation after 3 hours' incubation at 37°C. His method was adapted to the purpose of ultramicro procedure. A test tube rack suitable for this reaction was specially contructed.

CHOLINESTERASE[41]

Takahashi-Shibata's method[33] was modified by using m-dinitrophenol as indicator for the end point reaction in order to obtain a straight calibration line for reading enzyme activity. Acetylcholine (which is decomposed into acetic acid and choline by the enzyme) used as substrate is susceptible to spontaneous decomposition in aqueous solution. Accordingly, the calibration curve should be constructed at every analysis so that the error arising from the spontaneous degredation of the substrate in the standard tube may be minimized as well as the samples and standard tubes may be treated in the same way. After the first measurement of optical density (at 420 mμ) was over at the end of one hour's incubation, the color of alkaline m-nitrophenol in the solutions was erased by addition of a drop of 2N acetic acid. The optical density is measured for the second time. The fall of pH, namely ΔpH is evaluated which represents the enymatic activity is evaluated from the difference between the first and the second optical densities. This method compares quite well with Takahashi-Shibata's method.[33] Twenty λ of serum is sufficient for the analysis. (Figure 3-e).

TRANSAMINASE (GPT)

Mizobe's micromethod[34] which is a modification of Reitman-Frankel's method was adapted to ultramicroanalysis with 20 λ of serum[35]. Serum with activity higher than 100 Mizobe units was measured by shortening the reaction time instead of resorting to the conventional way of diluting the original serum. The results by this method were in good agreement with Mizobe's method.[34] (Figure 3-f).

ALKALINE PHOSPHATASE[42]

The principle of Kind-King's method[36] was followed. However, phenol liberated from the action of enzyme was brought into reaction with antiformin and sodium nitroprusside in the presence of $NH_4(OH)$ to form indophenol blue which was

suitable for colorimetry at 660 mμ. In Kind-King's method five steps are required before colorimetry, but in the present method the steps were reduced to only two. The new method compares quite well with Kind-King's micromethod.[36] (Figure 3-g).

AMYLASE

Our ultramicromethod belongs to saccharogenic method (like Somogyi method)[37] for the determination of amylase. Maltose and glucose liberated from the substrate (soluble starch) enzymatically was heated with 3,5-dinitrosalicylic acid to

Table 1. Ultramicro procedures employed for minipette system and their precision

Substance	Method	Amount of sample	Steps	Precision	
				Day to day variation	Duplicate determination
Glucose	o-Toluidine boric acid method[13]	20λ	1	± 3 %	< 2 %
Urea N	Direct diacetyl monoxime method[18]	20λ	1	± 3 %	< 2 %
N P N	Hypobromite phenosafranine method[20]	20λ	4	± 4 %	< 4 %
Bilirubin	Modification of Lathe-Hogg's method[23]	Total b: 20λ, Fraction: 100λ	1 2		< 2 % < 2 %
Hemoglobin	Cyanmet-hemoglobin method[25]	20λ	1	± 2 %	< 1 %
Protein	Biuret method[27]	20λ	1	± 2 %	< 1 %
Albumin	Chen's HABCA method[28]	20λ	1	± 2 %	< 2 %
Cholesterol	Zurkowski's method[29]	20λ	1	± 2 %	< 2 %
C C F T	Modified Martinek's method[32]	20λ	1		
Cholinesterase	Sasaki's m-nitrophenol method[41]	20λ	2	± 3 %	< 3 %
Transaminase GPT	Modification of Reitman-Frankel's method[35]	20λ	3	± 3 %	< 2 %
Alkaline phosphatase	Modification of Kind-King's method[42]	20λ	2	± 2 %	< 2 %
Amylase	Modification of Ujihira's method[38]	20λ	1	± 5 %	< 3 %
Leucine aninopeptidase	Takenaka-Takahashi's method[40]	20λ	1	± 3 %	< 2 %

produce a condensation product which was reddish brown in color. The colored solution was subjected to colorimetry at 540 mμ. Addition of thiosemicarbazide to the reagent (dinitrosalicylic acid) was helpful for the stabilization of the color.[38] Deproteinization is unnecessary. A good correlation was obtained between this ultramicromethod and Smith-Roe's amylocrastic method.[39] (Figure 3-h).

LEUCINAMINOPEPTIDASE

Takenaka-Takahashi's ultramicro method[40] was used without modification.

PRECISION AND EFFICIENCY OF THE ULTRAMICRO ANALYSIS BY USE OF MINIPETTE AND OUR COLORIMETER

In our laboratory the ultramicro methods which were described above have been in routine use since a year ago. Minipettes are used for taking reagents, and colorimetry is performed in the photoelectric colorimeter with drain system and trap. The sample required is usually 20 λ for each analysis.

In our experience the reproducibility of the anayses is excellent. (Table I). This is evidenced by the control chart which is depicted in Figure 4. Daily estimations underwent an irregular variations less distinctly in ultramicro analysis than in micro analysis.

Furthermore the minipette system has proved to be of better efficiency than the conventional micro analysis. About 1000 samples of blood serum for various kinds of analyses are dealt with by

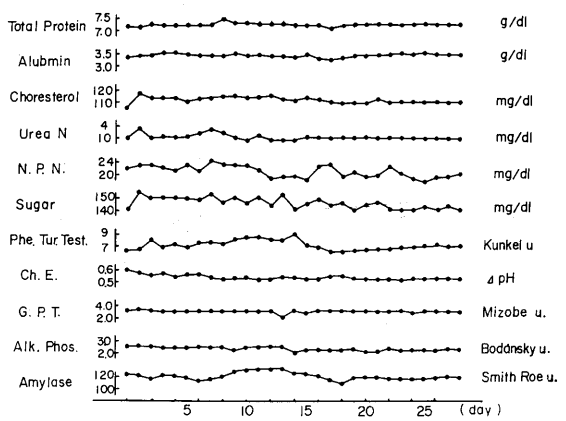


Fig. 4-a. Control chart of the ultramincro analysis by minipette system.

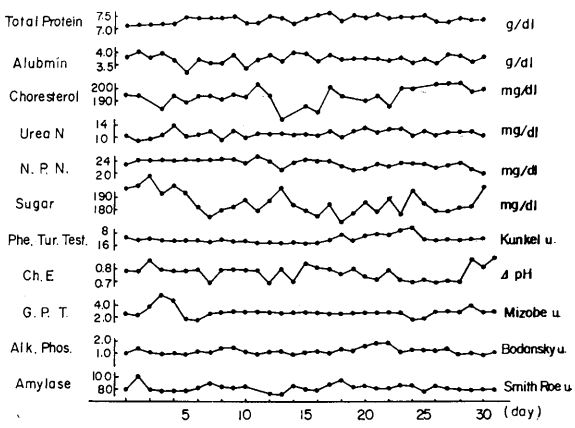


Fig. 4-b. Control chart of the conventional micro analysis.

medical technologists as small as five in number everyday. Works of this degree are not a significant burden for them.

Minipette system is really recommended for the clinical chemists who are interested in ultramicroanalysis.

Grateful acknowledgment is made to Prof. Susumu Shibata for his constant interest, suggestion and guidance in this investigation.

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