MICRO-ANALYSIS IN MEDICAL BIOCHEMISTRY

BY

E. J. KING

M.A. (McMaster), Ph.D. (Toronto)

PROFESSOR OF CHEMICAL PATHOLOGY IN THE UNIVERSITY OF LONDON AT THE BRITISH POSTGRADUATE MEDICAL SCHOOL

With 16 *Illustrations*

LONDON

J. & A. CHURCHILL LTD.

104 GLOUCESTER PLACE, W. 1

Reprinted 1947

First Edition . . . 1946
,, ,, Reprinted 1947

Printed in Great Britain

MICRO-ANALYSIS IN MEDICAL BIOCHEMISTRY

PREFACE

DESCRIPTIONS of procedures for micro-analysis of certain commonly determined blood constituents have been given in a series of communications emanating from this laboratory during several years (King, *et al.*, 1936 to 1944). These methods have proved their usefulness in research and routine laboratory work and, since their publication, have met with a considerable measure of adoption in this country and abroad. This fact has encouraged research into new methods of blood analysis and to the introduction, where possible, of modifications which enable the determinations to be carried out on small quantities of material. Some of the procedures described in the original papers have since been modified, and micro-methods for other constituents have been investigated. The improvements introduced in the procedures formerly described are embodied in the descriptions given here, together with directions for the additional methods.

The trend of analytical research during recent years has been increasingly in the direction of micro-modification of existing procedures and in innovations of principle and technique which make it possible to perform the necessary manipulations with small amounts of material and reagents. The well-known work of Pregl and of Emich needs only to be mentioned as an illustration of this tendency. Biochemistry has not lagged behind other branches of the science in exploiting micro-analysis, and many authors have published the results of work which has been taken advantage of in developing the present set of procedures. Some methods have been adopted without change ; in others we have introduced our own modifications, refinements, and innovations. In all, we have kept rigidly to sound principles of chemistry and technique and have avoided the adoption of proposals which seemed to us to rest on doubtful chemical ground.

Micro-analysis enjoys an advantage which is perhaps particularly worth stressing at the present time. The manipulations are carried out with only a fraction of the material

which is usually used. When an analysis of blood is performed for, say, uric acid, on 0·2 ml. of blood instead of the 2 ml. which are usually used, the saving in reagents is very considerable. Many of the reagents for blood analysis are becoming increasingly difficult to obtain in any quantity, and any economy in their use is both a saving and a convenience, and may even be a necessity. The amount of labour necessary for making up reagents is also greatly reduced. Within our own experience, solutions last many times as long as they did with methods we used previous to adopting micro-procedures, for the simple reason that much less of them is used for analyses on the micro-scale.

The accuracy of the micro-methods is usually beyond question, and biochemical methods carried out on small quantities of capillary blood have given at least as consistent and as accurate results as the larger scale procedures from which they usually sprang. The advantage of being able to omit any coagulant substance in the taking of the specimen needs no comment. The sample can be measured in most capillary blood pipettes with a high degree of precision and the possibility of obtaining abnormal proportions of cells and plasma when sampling an improperly mixed specimen of venous blood (a potent source of error not often appreciated) is avoided.

The level of some substances in arterial blood is different from and of greater physiological significance than the level in venous blood. This is notably so in the case of glucose. Arterial blood glucose is best estimated in capillary blood which gives the same value.

Micro-chemical methods of blood analysis are particularly useful in investigations which require the taking of frequent samples of blood. Determinations on capillary blood as compared with venous blood are less laborious for the investigator and are less inconvenient to the patient, who usually objects to numerous and elaborate venipunctures. A puncturing apparatus or a Hagedorn needle, together with a supply of capillary blood pipettes, is much easier to keep and to use than a supply of sterilized syringes and needles. The micro-methods described for whole blood have been developed

primarily for use with capillary blood, but they are of course applicable to samples of venous blood.

Among the methods of blood analysis will be found certain procedures, i.e., cholesterol, calcium and CO_2-combining power, which have been adopted, unaltered, from the published descriptions of the original authors. Although we have introduced no modification or improvement in these methods, we have felt it advisable to include them for the sake of completeness, and in order to have together a set of instructions which make it possible to execute all the determinations which are commonly asked for in routine laboratory investigations. For the same reason, the tests and estimations on CSF and faeces are included, and those urine examinations which are essential for the simpler physiological tests of function.

The functional tests given are described in sufficient detail to enable them to be executed with precision, but no great space is devoted to discussing their clinical significance. Likewise no attempt is made to give a complete statement of the amounts of the various substances present in blood in diseased conditions nor to describe their significance at any length. Brief mention is made only of those clinical conditions in which abnormal values are most commonly encountered.

My former colleagues, Dr. G. A. D. Haslewood, Mr. G. E. Delory and Dr. D. Beall, have had an intimate share in the development of the procedures presented in this book. On their ideas and efforts I have largely depended for the elaboration of new analytical principles and the modification of existing ones to the needs of the micro-techniques. To them and to other colleagues, assistants and students, my thanks are due, for their counsel, collaboration, and loyal support.

E. J. K.

CONTENTS

MICRO-ANALYSIS IN MEDICAL BIOCHEMISTRY

Chapter I

NORMAL VALUES

By " normal value " is meant the amount of a constituent present in the body fluid or excretion of a healthy human being. In fact, this amount varies over a range, and while most healthy persons can be included in a class having the accepted " normal " amount, some individuals are found to show divergent figures. Such exceptional individuals are often entirely " normal " in all other investigated respects. The judgment, therefore, of whether a given analytical figure is " normal " will depend on the experience and total data at the command of the interpreter of the result.

The values given below are taken from the literature and from our accumulated results. In most cases they are assumed to apply to the method of analysis given here. This assumption is generally the result of a direct comparison between the method given and a method which has been " standardized for normal human beings " ; in other cases, inspection of a large collection of determinations prompts the feeling that the findings of a modified method do not differ significantly from those " accepted." It must be emphasized, however, that in several cases (uric acid in blood, for example,) no reliable data of the range of " normal value " exist ; it is hoped that when more general agreement as to methods to be used has been attained, such data will be forthcoming. Blood values are for fasting persons.

TABLE 1.—NORMAL VALUES

	per 100 ml.
WHOLE BLOOD	
Urea	20–40 mg.
Non-protein nitrogen	25–40 mg.
Uric Acid	probably 2–4 mg.
"Creatinine"	1–2 mg.
Phosphate, inorganic (as P)—	
Adult	2–3 mg.
Child	4–5 mg.
Cholesterol	120–250 mg.
Sugar	60–100 mg.
Chloride (as NaCl)	450–510 mg.
PLASMA	
Total Protein	6·0–8·0 g.
Albumin	3·4–6·0 g.
Globulin	1·5–3·0 g.
(Ratio alb./glob. = 1·3–4·0)	
Fibrin	0·2–0·4 g.
Bilirubin	0·3–0·8 mg.
Chloride (as NaCl)	560–620 mg.
Sodium (as Na)	325–350 mg.
CO_2-combining power	55–75 ml.
Phosphate (as P)—	
ester	1 mg.
inorganic	2–3 mg.
lipide	4–7 mg.
Phosphatase, Acid	1–3 units
Alkaline	5–10 units
SERUM	
Calcium	9–11 mg.
Sodium	325–350 mg.
Potassium	16–20 mg.
CEREBRO-SPINAL FLUID	
Protein	20–40 mg.
Globulin (Pandy and Nonne Apelt Test)	absent
Chloride (as NaCl)	700–740 mg.
Sugar	60–100 mg.
Urea	15–30 mg.
Calcium	4–5 mg.
"Creatinine"	0·7–1·5 mg.
CO_2-combining power	55–65 vols.

	Percentage by weight of dried faeces.
FAECES	
A. Total fat	15–25
B. Unsoaped fat—	
(Neutral fat + Free fatty acid)	10–15
C. Free fatty acid	9–13
D. = A − B = Fatty acid present as soap	10–15
E. = B − C = Neutral fat	1–2

TABLE 2.—ABNORMALITIES IN COMPOSITION OF HUMAN BLOOD

Constituent	Clinical conditions in which high values (unless otherwise stated) are found
Plasma proteins (total)	Anhydremia. Low in nephritis with oedema (nephrosis), starvation.
Plasma albumin	Low in nephrosis.
Plasma globulin	Nephrosis, anaphylactic conditions.
CO_2-combining power	Alkalosis ($NaHCO_3$ administration, intestinal obstruction, over-breathing). Low in acidosis (diabetes, starvation, and severe nephritis).
Sugar	Diabetes, hyperthyroidism.
Non-protein N	Nephritis, eclampsia, intestinal obstruction, etc.
Urea	Nephritis, intestinal obstruction, etc.
Uric Acid	Nephritis, eclampsia, arthritis, gout.
Creatinine	Nephritis—only in severe cases above 4mg./100ml.
Chlorides (whole blood)	Nephritis, some cardiac conditions, eclampsia, prostatic obstruction, anaemia.
Chlorides (plasma)	Low in pneumonia, fever, diabetes; all cases of dehydration, such as gastro-intestinal disturbances associated with diarrhoea and vomiting.
Phosphates as P	Nephritis. Low in rickets.
Calcium (serum)	Hyperparathyroidism. Low in tetany (infantile), parathyroidectomy, severe nephritis, coeliac disease.
Cholesterol	Biliary obstruction, nephritis, nephrosis, diabetes, pregnancy. Low in pernicious anaemia.
Phosphatase	Generalized bone disease, obstructive jaundice.
Bilirubin	Jaundice.

CHAPTER II

PROCEDURES FOR WHOLE BLOOD

TAKING OF CAPILLARY BLOOD

Blood may be taken from a puncture in the ear or finger, but the most convenient place to obtain capillary blood is probably from the thumb over the bed of the nail. The part is wiped clean with a little ether or spirit and a stab of 1 to 2 mm. deep is made by means of a puncturing apparatus or Hagedorn needle. A piece of soft rubber tubing or of gauze is wrapped fairly tightly about the thumb above the knuckle. On flexing the thumb a free flow of blood is usually obtained. If the blood does not come easily, the rubber is released and the hand shaken in a downwards direction. This operation will ensure an adequate amount of blood when the tourniquet is replaced and the thumb flexed. The pipette is held horizontally with its point in the drop of blood issuing from the stab wound. The blood is allowed to run in exactly to the 0·2 ml. mark. The pipette is then wiped and the blood allowed to run into a 15 ml. conical centrifuge tube containing water or isotonic sodium sulphate solution, and by alternate blowing and sucking the pipette is washed several times with the solution.

TAKING OF VENOUS BLOOD

When several different estimations on whole blood are required it may be preferable to take a venous sample. Two ml. will usually suffice. The blood is withdrawn by a syringe from a vein in the antecubital fossa, according to the instructions given under *Plasma*, and is placed in a tube or screw-cap bottle containing a trace of potassium oxalate (the residue from a micro-drop of 30 per cent. dried in the tube).

UREA

Urea represents about 50 per cent. of the non-protein nitrogen of the blood. Normally there are between 20 and 40 mg. of urea present per 100 ml. High values are found in conditions associated with impaired renal function—particularly in chronic nephritis, but also in some cases of acute nephritis, prostatic obstruction, cardiac failure, etc.

PRINCIPLE

The sample of blood is digested with urease, and the urea thus converted into ammonia. After the removal of proteins, the colour produced by the ammonia with Nessler's reagent is compared colorimetrically with the colour produced under the same conditions with a standard ammonium chloride solution.

Direct Nesslerization does not lead to the production of cloudiness in the case of protein-free filtrates from unlaked blood. This is due to the fact that the sulphydryl substances, glutathione and ergothioniene, which produce turbidities with Nessler's reagent because of the insolubility of their mercury salts, are confined to the cells and do not appear in the filtrate, as is the case with filtrates of laked blood. Filtrates of unlaked blood have the further advantage that no ammonia is contributed to the determination through the action of the arginase of the red cells on the arginine contained in most commercial preparations of urease (see Addis, 1928). The use of zinc hydroxide as deproteinizing reagent eliminates a small amount of turbidity-producing substance contributed by most preparations of urease.

METHOD

0·2 ml. of blood is added to a centrifuge tube containing 3·2 ml. of isotonic sodium sulphate solution.

A " knife-point " (about 20 mg.) of Jack Bean meal is added, and the tube stoppered with a rubber bung, mixed, and incubated at 37° C. for 20 minutes. 0·3 ml. of zinc

sulphate solution and 0·3 ml. of 0·5 N-sodium hydroxide are added to precipitate the proteins. The mixture is well mixed by inversion after each addition and is then centrifuged. Two ml. of the supernatant fluid represent 0·1 ml. of blood.

Two ml. of the clear supernatant are treated with 5 ml. of ammonia-free distilled water and 1 ml. of Nessler's reagent. The solution is compared in a colorimeter with a " low " or " high " standard made up with 2 ml. or 5 ml. of the standard ammonium chloride solution (0·01 mg. of nitrogen per ml.), 5 ml. or 2 ml. respectively of water, and 1 ml. of Nessler's reagent*. The colorimetric comparison is facilitated by the use of a violet light filter.†

CALCULATION ‡

(1) " Low " standard :

$$\text{Blood urea}\ \S \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}02 \times \dfrac{100}{0{\cdot}1} \times 2{\cdot}14 \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 42{\cdot}8 \end{cases}$$

(2) " High " standard :

$$\text{Blood urea}\ \S \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}05 \times \dfrac{100}{0{\cdot}1} \times 2{\cdot}14 \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 107 \end{cases}$$

N.B.—1 mg. of nitrogen ≡ 2·14 mg. of urea

§ mg. per 100 ml. blood.

* If the blood urea is more than 150 mg. per 100 ml., the determination should be repeated with 1 ml. of supernatant (≡ 0·05 ml. blood).

† A Klett or Chance blue-violet light filter (obtainable from Messrs. Hearsons) is recommended for the colorimetric comparisons in the urea method, and for all comparisons of the Nessler yellow colour. This filter is particularly well suited for this comparison (see section on photometric measurement, p. 142). For photoelectric measurement of the colour a Chance blue-green or Ilford minus-red filter is suitable.

‡ The calculations given apply to results obtained with a Duboscq type colorimeter. For a photoelectric instrument certain modifications in the methods and in the form of the calculation are necessary. These are given on pp. 149, 150.

SOLUTIONS

Nessler's Reagent.—11·25 g. of iodine crystals are dissolved in a solution of 15 g. of potassium iodide in 10 ml. of water. 15 g. of mercury are added, and the mixture, kept cool in water, is shaken until the supernatant liquid has lost its yellow colour. This supernatant liquid is then decanted into a 100 ml. flask and a drop tested with 1 per cent. starch. If no colour is obtained, more iodine solution (prepared as above) is added until a drop of the mixture gives a faint reaction with starch.

The total solution is then diluted to 100 ml. and poured into 485 ml. of 10 per cent. sodium hydroxide. If the solution is turbid, it should be allowed to settle before use.

Standard Ammonium Chloride Solution (containing 0·01 mg. of nitrogen per ml.).—153 mg. of pure ammonium chloride are weighed out and dissolved in water. The volume is made to 100 ml.; 25 ml. of this solution with 10 ml. of N-sulphuric acid are diluted to 1 litre with distilled water.

Isotonic Sodium Sulphate.—Thirty g. of crystalline sodium sulphate ($Na_2SO_4.10H_2O$) are dissolved in water and made to 1 litre.

Zinc Sulphate.—Ten g. of crystalline zinc sulphate ($ZnSO_4.7H_2O$) are dissolved in water and made to 100 ml.

0·5 N-*Sodium Hydroxide.*—This should be accurately prepared, and should be titrated against the zinc sulphate. 10·8–11·2 ml. should be necessary to produce a permanent pink colour with phenolphthalein, when titrated into 10 ml. of zinc sulphate diluted with water.

NON-PROTEIN NITROGEN

The non-protein nitrogen containing substances of blood are urea (10–20 mg. N), uric acid (1–2 mg. N), creatinine (0·5–1 mg. N), amino-acid nitrogen (6–8 mg.), and substances such as glutathione and ergothioniene (5–10 mg. N per 100 ml. of blood). The normal range of non-protein (N.P.N.) is from 25–40 mg. per 100 ml. Increased values are found in the conditions showing a high blood urea.

PRINCIPLE

The proteins of laked blood or plasma are precipitated by tungstic acid. Part of the filtrate is digested with sulphuric acid until all the nitrogen is converted into ammonium sulphate. The ammonium salt is estimated colorimetrically with Nessler's solution, excess of which is used for the test in order to neutralize the sulphuric acid and give an alkaline medium.

METHOD

0·2 ml. of blood is laked with 3·2 ml. of water, or 0·2 ml. of blood in 3·2 ml. of isotonic sodium sulphate is laked by the addition of a drop of 1 per cent. saponin, followed by vigorous shaking. 0·3 ml. of 10 per cent. sodium tungstate and of $\frac{2}{3}$ N–H_2SO_4 are added and the mixture shaken and filtered or centrifuged.

One ml. of the filtrate (= 0·05 ml. of blood) is evaporated in a test-tube with 0·2 ml. of 50 per cent. sulphuric acid containing 1 per cent. SeO_2 until the liquid turns dark and white acid fumes are evolved. The addition of a small piece of porous pot prevents "bumping." Heating is continued until the mixture is colourless and for 3 or 4 minutes more. To the cooled solution are now added 5 ml. of water and, after thorough mixing, 3 ml. of Nessler's solution. The colour produced is compared in the colorimeter with the "low" or "high" standard used in the determination of blood urea.

CALCULATION

(1) "Low" standard :

$$\text{N.P.N.*} \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}02 \times \dfrac{100}{0{\cdot}05} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 40 \end{cases}$$

* mg. per 100 ml. of blood or plasma.