MICRO-ANALYSIS IN MEDICAL BIOCHEMISTRY

BY

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PREFACE TO THE SECOND EDITION

THE generous reception of this book in 1946 has encouraged me to prepare a revised edition, which has been brought up-to-date, modified and somewhat enlarged. The general plan of the first edition appeared reasonably satisfactory, both for teaching purposes and for a manual of methods for biochemical research and routine analyses; and it has been retained. A first chapter dealing with 'normal values' presents the results of recent work in establishing what are the statistically probable ranges of biochemical constituents of the blood, when determined by the methods described in this book and carried out under routine laboratory conditions. In this I have been fortunate in having the collaboration of my colleague Dr. I. D. P. Wootton. The following chapters deal with the collection and biochemical investigation of blood, urine, etc., tests of function, pH, preparation of volumetric solutions, and those techniques of colorimetry and photometry which are somewhat special to biochemical practice. New material introduced includes: ketosteroids, formaldehyde-stable acid phosphatase, the insulin *plus* glucose test, effective renal plasma flow and glomerular filtration rate, fat balance, and several new, more micro and modified analytical methods.

In the description of colorimetric procedures, the assumption has been made that photoelectric instruments will be used by the majority of workers; and the calculations for photoelectric readings have been placed first. In each instance, however, the method of readings and the form of calculation for the Duboscq type of colorimeter have also been given, together with an indication of any necessary modification in the procedure. The new grey-wedge photometer, which has proved most successful as an accurate, though simple, field instrument for determining hæmoglobin, has been included in the final chapter on colorimetry and photometry, together with instructions as to how it may be

used as a general instrument for all colorimetric methods of analysis.

In order to make room for new procedures, without unduly increasing the size of the book, some material of the first edition has been omitted, e.g., the artificial standards and methods which were developed during the war for the easy estimation of blood, C.S.F. and urine constituents in field laboratories.

It has always been intended that this should be a book to use, rather than a text-book to read; and all statements and descriptions have been kept to the minimum length compatible with clarity of instruction. For this reason the brief statements of the use that may be made of each determination in the investigation of disease may appear cryptic and inadequate to the clinician. But the few criticisms which have reached me on this score are greatly outweighed by the approval of the many laboratory workers, clinical pathologists and students, who have found these brief reminders of biochemical abnormalities a ready and easy means of refreshing their memories on matters about which they have previously read extensively in text books of clinical medicine, but have in part forgotten.

To my colleagues and friends, who have generously contributed of their time and thought to the testing and selecting of new methods and the checking of modified procedures, my sincere thanks are due. In this regard I am especially grateful to Prof. R. H. A. Plimmer, Dr. I. D. P. Wootton, Dr. W. Klyne, Dr. G. M. Bull, Dr. D. A. K. Black and Dr. S. P. V. Sherlock; and to Miss V. Bentley, Miss M. Cox, Dr. R. M. Haslam and Miss V. R. Pash for their careful checking of proofs and preparation of the index.

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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 in Table 1 are taken from the literature and from our accumulated results. In most cases they apply to the method of analysis given here. In others they are the result of a direct comparison between the method given and a method which has been 'standardized for normal human beings.' Blood values are for fasting persons.

The figures for whole blood, plasma and serum are based on a survey by Wootton, Maclean Smith and King (1950) of about 80 normal adults aged 20 to 50 years. It would have been convenient to express results as an average normal figure with a standard deviation. The plotted results for blood values have usually given a 'skew' type of distribution curve, however; and they have been listed, therefore, in terms of a range. 80 per cent of the normal values given fall between the upper and lower 10 per cent limits, and 98 per cent between the upper and lower 1 per cent limits. Thus 1 per cent of normal subjects have blood urea values lower than

TABLE 1

Normal Values

	mg. per 100 ml.*			
	Lower 1% level	Lower 10% level	Upper 10% level	Upper 1% level
WHOLE BLOOD				
Urea	12	16	35	47
Non-protein nitrogen	25	29	43	51
Uric Acid	0.6	1.6	3.9	4.9
Creatinine	0.1	0.1	1.2	2.6
Phosphate, inorganic (as P)—				
Adult		2	3	
Child		4	5	
Cholesterol	115	140	215	265
Sugar	55	68	96	109
Chloride (as NaCl)		450	510	
PLASMA				
Total Protein	6.3	6.7	7.7	8.2 g.
Albumin	4.0	4.4	5.8	5.7 g.
Globulin	1.5	1.9	2.8	3.0 g.
(Ratio alb./glob. = 1.3–3.6)				
Fibrin		0.2	0.4	g.
Bilirubin	0.1	0.1	0.5	0.8
Chloride (as NaCl)	581	598	620	632
Sodium (as Na)	306	316	340	350
CO_2-combining power		56	74 vol.	
Phosphate (as P)—				
ester		1	2	
inorganic		2	3	
lipid		7	10	
Phosphatase, Acid	0.8	1.2	3.1	4.6 units
Acid, formol stable	0	0	2.1	4.1 units
Alkaline	3.3	4.5	9.5	12.9 units
Amylase	71	91	168	209 units
SERUM				
Calcium	9.1	9.6	10.9	11.4
Sodium	307	318	342	358
Potassium	13.5	15.1	19.6	21.7

CEREBRO-SPINAL FLUID	mg. per 100 ml.
Protein	20–40
Globulin (Pandy and Nonne Apelt Test)	absent
Chloride (as NaCl)	700–740
Sugar	60–100
Urea	15–30
Calcium	4–5
Creatinine	0.7–1.5
CO_2-combining power	55–65 vols.

FÆCES	Percentage by weight of dried fæces
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

*Except where stated otherwise.

12 mg., 9 per cent between 12 and 16 mg., 80 per cent between 16 and 35 mg., 9 per cent between 35 and 47 mg., and 1 per cent over 47 mg. per 100 ml. In clinical practice any single

TABLE 2

Abnormalities in Composition of Human Blood

Constituent	Clinical conditions in which high values (unless otherwise stated) are found
Amylase	Acute pancreatitis.
Bilirubin	Jaundice.
Calcium (serum)	Hyperparathyroidism. Low in tetany (infantile), parathyroidectomy, severe nephritis, cœliac disease.
Chlorides (whole blood)	Nephritis, some cardiac conditions, eclampsia, prostatic obstruction, anæmia.
Chlorides (plasma)	Low in pneumonia, fever, diabetes ; all cases of dehydration, such as gastro-intestinal disturbances associated with diarrhœa and vomiting.
Cholesterol	Biliary obstruction, nephritis, nephrosis, diabetes, pregnancy. Low in pernicious anæmia.
CO_2-combining power	Alkalosis ($NaHCO_3$ administration, intestinal obstruction). Low in acidosis (diabetes, starvation, and severe nephritis).
Creatinine	Nephritis—only in severe cases above 4mg. per 100 ml.
Non-protein N	Nephritis, eclampsia, intestinal obstruction, etc.
Phosphatase (alkaline)	Generalized bone disease, obstructive jaundice.
Phosphatase (acid)	Carcinoma of the prostate.
Phosphates	Nephritis. Low in rickets.
Plasma proteins (total)	Anhydremia. Low in nephritis with œdema (nephrosis), starvation.
Plasma albumin	Low in nephrosis.
Plasma globulin	Nephrosis, anaphylactic conditions, hepatitis.
Potassium	Addison's disease, terminal nephritis.
Sodium	Low in Addison's disease.
Sugar	Diabetes, hyperthyroidism.
Urea	Nephritis, intestinal obstruction, cardiac failure, etc.
Uric Acid	Nephritis, eclampsia, arthritis, gout.

result falling *outside* the 10 per cent limits is considered suspicious ; a result which is *outside* the 1 per cent limit is almost certainly abnormal.

Milli-equivalent values per litre of blood are listed for many constituents in the individual sections for each substance.

In studies of acid-base balance it is often desirable to express the concentrations of those constituents of plasma

TABLE 3

Approximate Average Daily Composition of Human Urine

	mg. per 100 ml.	g. per 24 hr.	As nitrogen g. per 24 hr.	Milli-equivalents per 24 hr.
NITROGENOUS CONSTITUENTS				
Urea	2000	30	14	
Ammonia	50	0.8	0.7	50
Creatinine	100	1.5	0.56	
Hippuric Acid	30	0.5	0.04	3
Amino-Acids	400	6.25	1	
Uric Acid	25	0.4	0.17	2
Urochrome and other pigments				
SULPHUR-CONTAINING CONSTITUENTS				
Inorganic sulphates (as H_2SO_4)	120	1.8		36
Ethereal sulphates, e.g. indican	20	0.3		2
'Neutral' sulphur compounds, e.g. NaCNS	20	0.3		
OTHER CONSTITUENTS				
Organic				
Oxalic acid	1	0.02		0.5
Carbonic Acid				
Aromatic hydroxyacids, e.g. *p*-hydroxyphenylacetic				
Inorganic				
Phosphate (as P)	110	1.7		100
Chloride (as NaCl)	800	12		
Chloride (as Cl)	485	7.3		200
Sodium	670	10		430
Potassium	170	2.5		65
Calcium	18	0.2		10
Magnesium	18	0.2		16
Water		1500		

which act as acids or bases in terms of equivalents, so that the 'total acid' of the plasma may be compared with the 'total base,' thereby demonstrating if there is a preponderance of acid over base, or *vice versa*, i.e. whether the 'acid-base

balance' favours an acid or an alkaline state. It may be convenient, therefore, to express the results for the acid radicles [chloride, carbon dioxide (bicarbonate), phosphate, sulphate, proteins and organic acids] and the basic (sodium, potassium, calcium, magnesium) as milli-equivalents per litre of plasma ; the equivalent, that is, of the amount of acid or base which they represent, or are capable of neutralizing. The plasma proteins, for instance, act as weak acids, and neutralize a certain quantity of base ; and it is possible to express their concentration, as one would for an acid, in terms of the amount of the base they neutralize. This is done by dividing the concentration of protein (in mg. per litre) by the average equivalent weight of the plasma proteins, i.e. by that fraction of their molecular weight which represents the amount which would neutralize one milli-equivalent of sodium hydroxide (i.e. 1 litre of N/1000 NaOH). This is most easily done by the use of a factor : g. protein (per 100 ml.) $\times 2.43$ =milli-equivalents per litre. With simple monovalent ions like sodium and chloride the mg. per litre are divided by the atomic weight ; with divalent ions (e.g. calcium) by half the atomic weight.

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, Hagedorn needle or sharp fragment of glass. 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. 2 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* (p. 37), 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 at about 100°C.) or 1 drop of heparin (1000 units per ml.).

DEPROTEINIZATION OF BLOOD

Urea, sugar and several other substances are present in both the plasma and cells of blood ; but they diffuse easily out of the cells when the blood is diluted. Since there are definite advantages, for several analytical purposes, in leaving the cells intact so that interfering substances contained in them will not be liberated and appear in the protein-free extract of the blood, the custom of diluting the blood with an isotonic liquid, rather than laking it with water, has been adopted (Herbert and Bourne, 1930). Isotonic sodium sulphate solution is used, rather than sodium chloride, because the sulphate ion interferes less in the chemical reactions used than chloride does.

Many deproteinizing agents have been described. Those used here have been selected to fit the individual case ; and always for some special reason. Thus, copper tungstate is employed in the blood-sugar method because it is the most efficient means of eliminating the small amount of non-sugar reducing substances in the plasma, i.e. in the unlaked diluted blood. But it cannot be used for the colorimetric urea method, because of interference with Nessler's reagent ; nor in the non-protein nitrogen procedure, since it precipitates some of the non-protein nitrogen-containing substances. Similarly, the zinc hydroxide deproteinization used for urea is useless for sugar methods which are based on alkaline copper reagents, because of a depression of the activity of the copper reagent by small amounts of zinc which are present in the filtrate (King, Haslewood and Delory, 1937*b*). Nor should it be used for creatinine or phosphate, since it precipitates part of these constituents, and low results would be obtained. For substances like phosphate and sulphonamides it is necessary to use a fairly strongly acid protein-precipitating agent, in order to extract them completely ; or to keep them in an analysable condition as with ascorbic acid.

UREA

Urea represents about 50 per cent of the non-protein nitrogen of the blood. Normally there are between 16 and

35 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, cardiac failure, prostatic obstruction, intestinal obstruction, 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 or with a standard urea solution treated with urease.

Direct Nesslerization should not lead to the production of cloudiness in the case of protein-free filtrates from unlaked blood. The sulphydryl substances, glutathione and ergothioneine, 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

Test. 0.2 ml. of blood (or of plasma) is added to a centrifuge tube containing 3.2 ml. of isotonic sodium sulphate solution. (0.2 ml. of standard urea solution, similarly treated, is a useful check on the method, including the activity of the urease.)

A 'knife-point' (about 20 mg.), or small 'spoon' (5 mm. of glass tube, 3 mm. internal diam., fused at right angles on the end of a glass rod), of Jack Bean* meal is added, and the tube

* The 'Arlco' Jack Bean meal (Arlington Chemical Company, Yonkers, New York) is a very suitable preparation. Others have appeared to be not as potent in urease activity, or as good in keeping quality. A crushed "urease tablet" may be similarly used. (N.B. The use of too much urease may lead to clouding when the Nessler reagent is added.)

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, or filtered through a small (7 or 5.5 cm.) paper. 2 ml. of the supernatant fluid (representing 0.1 ml. of blood) are treated with 5 ml. of ammonia-free distilled water and 1 ml. of Nessler's reagent.

Standard. The solution is compared in a colorimeter with a 'low' or 'high' standard made up with 2 ml. and 5 ml. of the standard ammonium chloride solution (0.01 mg. of nitrogen per ml.), 5 ml. and 2 ml. respectively of water, and 1 ml. of Nessler's reagent.

Blank. For photoelectric measurement of the colour it is advisable to make the zero setting of the instrument with the solvent used in the estimation, usually water. A 'reagent blank,' consisting of 7 ml. of water and 1 ml. of Nessler, should be read at the same time as the standard and test, and its reading subtracted from those of test and standard. Alternatively, the zero setting may be made with the 'blank' when it will not be necessary to subtract from the readings of test and standard.

The colorimetric comparison is made with the use of an Ilford blue (622) or a violet light filter (621, see p. 203).*

CALCULATION

Photoelectric Colorimeter.

(1) 'Low' standard :—

$$\text{Blood urea}\dagger \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.02 \times \dfrac{100}{0.1} \times 2.14 \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 42.8 \end{cases}$$

* If the test reads more than double the standard, the colorimetric comparison should be repeated with 0.5 ml. of supernatant ($\equiv$0.025 ml. blood) *plus* 6.5 ml. of water ; (or 2 ml. of test diluted immediately with 6 ml. of blank).

† mg. per 100 ml. blood.

(2) 'High' standard :—

$$\text{Blood urea}^* \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.05 \times \dfrac{100}{0.1} \times 2.14 \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 107 \end{cases}$$

N.B.—1 mg. of nitrogen≡2.14 mg. of urea

* mg. per 100 ml. blood.

Duboscq Colorimeter. The calculation is the same as the above, except that the ratio of readings is inverted to Reading of standard/Reading of test, since the colour is inversely proportional to the concentration with the Duboscq type of colorimeter, instead of directly proportional as with the photo-electric. The 'blank' is not used.

SOLUTIONS

Nessler's Reagent. 11.3 g. of iodine crystals are weighed on a rough balance and dissolved in a solution of 15 g. of potassium iodide in 10 ml. of water. To 15 g. of mercury in a glass-stoppered reagent bottle is added most of this solution, 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 of the iodine solution 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. The solution, if turbid, should be filtered or allowed to settle before use, and should be kept in a bottle with a rubber stopper.

Standard Ammonium Chloride Solution (containing 0.01 mg. of nitrogen per ml.). 153 mg. of pure ammonium chloride (dried in a desiccator) are dissolved in water and the volume made to 100 ml. 25 ml. of this solution with 10 ml. of N-sulphuric acid are diluted to 1 litre with distilled water.

Standard Urea Solution. 100 mg. in 100 ml. water, preserved with a drop of chloroform and kept in the cold.

Isotonic Sodium Sulphate. 30 g. of crystalline sodium sulphate ($Na_2SO_4.10H_2O$), or 13.2 g. anhydrous Na_2SO_4, are dissolved in water and made to 1 litre.

Zinc Sulphate. 10 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 (see p. 187), 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. It is best kept in a wax bottle.

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 nitrogen (N.P.N.) is from 29–43 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 trichloroacetic 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

Test. 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. 0.6 ml. of 25 per cent trichloroacetic acid is added and the mixture shaken and centrifuged or filtered through a small (5.5 or 7 cm.) paper.

1 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. (See p. 42 regarding the method of heating.) 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.