MICRO-ANALYSIS IN MEDICAL BIOCHEMISTRY

THIRD EDITION

By

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PREFACE

I HAVE been joined in the third edition of 'Micro-Analysis in Medical Biochemistry' by my friend and colleague, Dr. I. D. P. Wootton, whom I welcome as a co-author. As a result of his efforts and researches, several new types of procedure have been introduced into the routine practice of this laboratory, and these have been included in new chapters. They are: a discussion of the control of laboratory accuracy and a system of quality control; electrophoresis of plasma proteins; techniques of metabolic balance studies; flame photometry of sodium, potassium and calcium; radioactive isotope tests.

New procedures include the following: free and ester cholesterol by a ferric chloride method; uric acid by uricase; true creatinine by adsorption and elution; the Markham micro-Kjeldahl method; biuret method for plasma proteins; a potentiometric chloride method; phosphatase by an amino-antipyrine determination of the liberated phenol; antipyrine for total body water; ethylenediamine tetra acetate determination of calcium; serum iron; the zinc sulphate turbidity test; proteolytic activity of fæces; paper chromatography of urinary sugars; lead and mercury in urine and fæces; the tubeless test meal; the pyruvate metabolism test; the water concentration test; inulin clearance (instead of thiosulphate); and a vitamin A absorption test. These have come into widespread use during the last five to ten years, and are now current practice at this School.

The chapter on colorimetric and spectrophotometric analysis has been largely rewritten. The sections on the Duboscq and simple colorimeters have been omitted and that on photoelectric practice expanded. Spectrophotometers are coming into increasing use, and an explanation and description of them has been given at some length, since it appears that a large proportion of analytical practice will centre round them in the next few years. These instruments extend photometric measurements into the ultraviolet and infrared, and procedures which measure light absorption in regions of the spectrum

other than the visible can be expected to come into increasing use. Such methods described in this edition include: barbiturates, uric acid and vitamin A in the ultraviolet; oxy- and reduced hæmoglobin and carboxyhæmoglobin in the near infrared.

For all colorimetric methods, both new and those carried forward from the previous edition, the use of the Duboscq colorimeter and the resulting calculations have been dropped, and only the photoelectric included. This step appears justified because of the almost universal adoption of the latter type of instrument. On a similar score, the expression of all electrolyte concentrations is now given in milliequivalents, a practice which should have come into use long ago, but cannot even yet be considered to be world-wide.

Emphasis has been maintained on the micro aspects of analysis, without going into the field of what might be called the 'ultra-micro'. Such procedures, it is felt, have their principal use in the biochemistry of children's diseases, and not in ordinary biochemical practice. The quantities of blood used for most of the procedures have been cut from 0·2 ml. to 0·1 ml. (in some cases to 0·05 ml.), and this has been found to be particularly advantageous where finger-prick specimens are employed. Many of the methods are the same as, or only slightly modified from, those which were described in the former editions; they have been well proved by years of use in both routine and teaching laboratories. The book continues to represent the routine laboratory practice of chemical pathology at the Postgraduate Medical School. Although many 'research' investigations have had to be omitted, because of a desire to keep the book to the reasonably small dimensions required in a laboratory manual, most of what we do is described here. As before, we have assumed a reasonable knowledge on the part of the user of the fundamentals of chemical and analytical theory and practice.

My sincere thanks are due to others of my colleagues, besides my co-author, Dr. Wootton, and these include: Dr. W. Klyne, Dr. I. MacIntyre, Dr. D. J. R. Laurence, Dr. J. G. B. Fenton, Dr. J. Pryce, Dr. J. E. S. Bradley, Dr. R. Fraser and Dr. M. D. Milne. Thanks are renewed to Dr.

D. A. K. Black and Professor G. M. Bull for help with sections carried forward from the last edition. To Miss P. R. N. Kind I am especially grateful for her skilled and painstaking assistance in the task of rewriting, editing and proof-reading; and to Miss Beryl Currie and Mrs. Margaret Sutton for their unstinted and expert help with the typing of script and reading of proofs.

E. J. KING.

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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.

TABLE 1

Normal Values

	Lower 1%	Lower 10%	Upper 10%	Upper 1%	Distribution
WHOLE BLOOD					
Urea (mg. per 100 ml.)	12	16	35	47	Lognormal
Non-protein nitrogen (mg. per 100 ml.)	25	29	43	51	Lognormal
Uric acid (mg. per 100 ml.)	0·6	1·6	3·9	4·9	Normal
Creatinine (mg. per 100 ml.)	0·1	0·1	1·2	2·6	Lognormal
Phosphate (inorganic as P) (mg. per 100 ml.)	2·0	2·4	3·5	3·9	Normal
Cholesterol (mg. per 100 ml.)	115	140	215	265	Lognormal
Glucose (mg. per 100 ml.)	55	68	96	109	Normal
Chloride (as NaCl) (mg. per 100 ml.)	425	454	526	555	Normal

	Lower 1%	Lower 10%	Upper 10%	Upper 1%	Distribution
SERUM AND PLASMA					
Sodium (m.eq. per litre)	133	137	148	152	Normal
Potassium ,, ,,	3·5	3·9	5·0	5·6	Lognormal
Calcium ,, ,,	4·5	4·8	5·4	5·7	Normal
Chloride ,, ,,	99	101	106	108	Normal
CO_2-combining power (m.eq. per litre)	24	25	29	31	Normal
Phosphate (inorganic) (m.eq. per litre)	1·4	1·7	2·4	2·6	Normal
Total protein (m.eq. per litre)	15·4	16·4	18·8	20·0	Normal
Total protein (g. per 100 ml.)	6·3	6·7	7·7	8·2	Normal
Albumin (g. per 100 ml.)	4·0	4·4	5·3	5·7	Normal
Globulin ,, ,,	1·5	1·9	2·8	3·0	Negative skewness
Fibrin ,, ,,	0·1	0·2	0·4	0·5	Normal
Bilirubin (mg. per 100 ml.)	0·1	0·1	0·5	0·8	Lognormal
Cholesterol (mg. per 100 ml.)	123	153	260	324	Lognormal
Phosphate (inorganic) (mg. per 100 ml.)	2·4	2·9	4·1	4·5	Normal
Phosphate (ester) (mg. per 100 ml.)	0·0	0·1	0·6	1·7	Lognormal
Phosphate (total acid soluble) (mg. per 100 ml.)	2·7	3·2	4·3	4·7	Normal
Phosphate (lipid) (mg. per 100 ml.)	7·0	8·3	12·6	14·9	Lognormal
Phosphatase (alkaline) (K.A. units per 100 ml.)	3·3	4·5	9·5	12·9	Lognormal
Phosphatase (acid) (units per 100 ml.)	0·8	1·2	3·1	4·6	Lognormal
Phosphatase (formol-stable) (units per 100 ml.)	0·0	0·0	2·1	4·1	Lognormal
Amylase (units per 100 ml.)	71	91	163	209	Lognormal

CEREBRO-SPINAL FLUID	mg. per 100 ml.	m.eq. per litre
Protein	20–40	—
Globulin (Pandy and Nonne Apelt Test)	absent	—
Chloride (as NaCl)	700–740	120–126
Sugar	60–100	—
Urea	15–30	—
Calcium	4–5	2–2·5
Creatinine	0·7–1·5	—
CO_2-combining power	55–65 vols.	25–29

FÆCES	Percentage by weight of dried fæces
A. Total fat	10–25
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

The figures for whole blood, plasma and serum are based on surveys by Wootton, Maclean Smith and King (1950) and Wootton and King (1953) 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 12 mg., 9 per cent between 12 and 16 mg., 80 per

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	Hyperparathyroidism, invasive bone tumours including sarcoids, myelomatosis. *Low* in tetany (infantile), parathyroidectomy, renal failure, cœliac disease.
Chloride	In general this reflects the plasma sodium level except in conditions where there is an abnormality in acid base balance.
Cholesterol	Biliary obstruction, nephrotic syndrome, diabetes, pregnancy, myxœdema. *Low* in thyrotoxicosis.
CO_2-combining power	Alkalosis ($NaHCO_3$ administration, pyloric stenosis, potassium deficiency), respiratory acidosis. *Low* in acidosis (diabetic ketosis, starvation and renal failure).
Creatinine	Renal failure.
Non-protein N	See urea.
Phosphatase (alkaline)	Generalized bone disease, obstructive jaundice.
Phosphatase (acid)	Carcinoma of the prostate.
Phosphate	Renal failure. *Low* in rickets.
Plasma proteins (total)	*Low* in nephrotic syndrome.
Plasma albumin Plasma globulin	See pages 50, 116.
Potassium Sodium	See pages 61, 105, 193, 194.
Sugar	Diabetes, hyperthyroidism.
Urea	Renal failure, intestinal obstruction, cardiac failure, hæmatemesis.
Uric acid	Renal failure, gout.

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 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.

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	40	0·6	0·1	
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	13	0·2		10
Magnesium	13	0·2		16
Water		1500		

When considering the concentrations of ionic constituents of blood or urine, it is convenient to express them in terms of chemical equivalents so that variations in one ion can be

compared directly with variations in another. It is customary, 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.) $\times 2 \cdot 43$ = 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, i.e. by use of the equation,

$$\text{milli-equivalents (m.eq.) per litre} = \frac{\text{mg. per 100 ml.} \times 10 \times \text{valency}}{\text{atomic weight}}$$

To calculate m.eq. per litre, divide mg. per 100 ml. by

Sodium	2·3
Potassium	3·9
Calcium	2·0
Chloride (as mg. NaCl)	5·85
(as mg. Cl)	3·55
CO_2-combining power (as vols. per 100 ml.)	2·24
Phosphate (as mg. P)	1·72
Protein (as g. per 100 ml.)	0·41

CHAPTER II

CONTROL OF LABORATORY ACCURACY

A NUMBER of surveys of laboratory accuracy have been conducted during recent years. The usual procedure was to distribute to several laboratories identical specimens of, say, blood or freeze-dried plasma. Each laboratory then analysed its specimen for the various constituents and the results were compared. In every survey, gross differences were observed in the values obtained. Thus Wootton and King (1953) reported that the highest blood urea result was four times the lowest, and similar variation was found with other constituents.

The very large differences between the results from various hospitals indicate that many clinical laboratories are less accurate than is generally supposed; and, indeed, their results may not be sufficiently precise to serve the purpose for which they are required. With this in mind, we have established a system of 'quality control' in our own laboratories which has, we believe, made a very great contribution towards maintaining an acceptable standard. Analyses of clinical specimens are usually done in batches, which may be put through twice daily or less frequently depending on the demands. With each batch, the analyst concerned includes a control solution whose exact composition is unknown to him. In due course he reports the result obtained in this control solution; it is compared with the true value, and the batch of analyses is accepted or rejected depending on whether the error of the control result is less or greater than that prescribed for the method.

In the majority of methods described in this book, comparison of some sort is made between the specimen under analysis and a known standard solution. It might be thought that the simultaneous examination of the known standard would be a sufficient safeguard against error. It is true that the standard sometimes indicates that something is amiss, but experience has shown that the result of the unknown

control is a more sensitive indicator that all is well. The control solution is treated exactly like the test specimens and takes part in all the analytical stages. It is particularly important that the analyst should not know the true result of the control solution. This does not imply any suspicion of dishonesty, but ignorance of the result is the only way of avoiding the powerful 'unconscious bias' which otherwise operates. It follows that the control solution must be varied each time to prevent the analyst from learning the result.

Setting up a Quality Control System. Before a system can be operated, it is necessary to decide the limits of error which will be acceptable. The limits must not be so narrow as to cause the rejection of a large proportion of the batches done, neither should they be too wide. The use for which the results are required will also influence the permitted error. It may be necessary to investigate the precision of a method in routine use; this can be done by repeatedly analysing one or more samples and examining the results obtained. However, it must be emphasized that a reliable indication of the scatter of a method will not be obtained if a single analyst carries out many determinations on the same specimen, knowing what he is doing. Under these circumstances the scatter will be less than it should be.

The limits which we ourselves use for certain common determinations are given below. They have been satisfactory for routine clinical use although the permitted errors may appear to be larger than would be expected. They were determined by an unbiased investigation and are, we believe, representative of good routine practice.

A SYSTEM FOR PLASMA SODIUM, POTASSIUM AND CHLORIDE

The control solution for each batch is selected from one of ten. Solutions I–V are made by dissolving the stated quantities of pure dry salts in distilled water. Solutions VI–X are mixtures as shown.

TABLE 4

Control Solutions

The limits allowed on results are: sodium ± 2, potassium ± 0·2, chloride ± 2 m.eq. per litre

Control Solution	Concentration (g. per litre) NaCl	KCl	Na_2CO_3	True value (m.eq. per litre) Na	K	Cl
I	5·8460	0·2982	2·1202	140	4·0	104
II	5·2614	0·1640	2·8622	144	2·2	92
III	4·0922	0·4921	4·2404	150	6·6	77
IV	6·8983	0·3803	—	118	5·1	123
V	5·7875	0·2683	2·0142	137	3·6	103
VI	Equal volumes of I and II			142	3·1	98
VII	,, ,, ,, II ,, III			147	4·4	84
VIII	,, ,, ,, I ,, IV			129	4·6	114
IX	,, ,, ,, IV ,, V			128	4·4	113
X	,, ,, ,, III ,, V			144	5·1	90

A SYSTEM FOR PLASMA BICARBONATE, BLOOD UREA AND BLOOD GLUCOSE

A stock solution of each substance is prepared. To prepare a control solution, 1 ml. of the stock solution is mixed with a randomly-chosen volume of distilled water (between the limits 2–20 ml.). The true value of the sample can readily be calculated, but it is convenient to prepare a table showing the concentrations contained in various mixtures of stock solution and water.

The limits allowed on results are: bicarbonate ±1 m.eq. per litre; urea ±10%; glucose ±10%. The values of urea and glucose in blood, and hence the values of control solutions, cover so wide a range that the error is better expressed as a percentage of the true value rather than in absolute terms.

Stock carbonate solution (200 m.eq. HCO_3^- per litre) 21·2 g. of pure dry anhydrous sodium carbonate are dissolved in water and made to 1 litre.

Stock urea solution (1000 mg./100 ml.) 1·000 g. of dry urea is dissolved in water and made to 100 ml. The solution is kept in the refrigerator.

Stock glucose solution (1000 mg./100 ml.) 1·000 g. of dry glucose is dissolved in saturated benzoic acid solution and made to 100 ml.

RECORDING OF RESULTS

It is instructive to plot the errors of the control results on a control chart as shown in Fig. 1. If a method is working well, the errors will be within the limits allowed and will be equally distributed above and below the line representing the true value. The total error of a control determination, plotted on the chart, represents the end-result of numerous small deviations from ideal conditions which have occurred at different stages during the analysis. The error, therefore, obeys

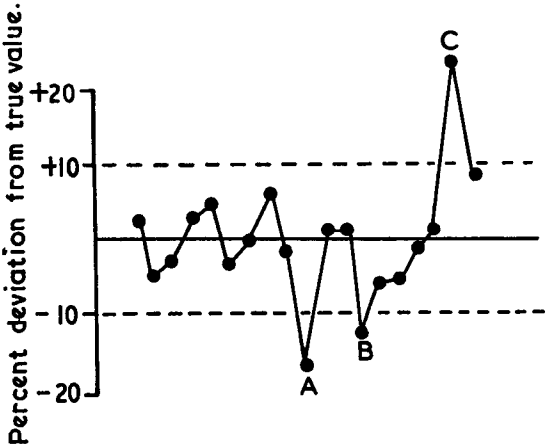

FIG. 1. Portion of control chart for urea determinations.

statistical laws and occasional cases will occur where the error is outside the limits and no cause can be found. No action is required except re-analysis of the batch.

If, however, repeating the control determination continues to give a result which is outside the limit of error, investigation is needed to discover what is amiss. Fig. 1 illustrates the type of situation which may be found. At point A, a damaged light filter in the photoelectric colorimeter had just been changed for another, of the same make and type. After many other possibilities had been excluded, the filter was examined and its spectral transmission was found to be incorrect. At point B it was discovered that a new analyst was