PRACTICAL
CLINICAL
BIOCHEMISTRY

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HAROLD
VARLEY

SECOND
EDITION

PRACTICAL CLINICAL BIOCHEMISTRY

BY

HAROLD VARLEY

M.Sc., F.R.I.C.

Biochemist, Manchester Royal Infirmary.
Lecturer in Clinical Pathology,
Manchester University.

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PREFACE

THE rapid development of clinical biochemistry has been an outstanding feature of medicine during the past twenty to thirty years. The present book is a survey of the whole field of this subject from the standpoint of workers in hospital laboratories. It is hoped that it will appeal particularly to registrars training in clinical pathology, to hospital biochemists, and to laboratory technicians. The book should be especially useful to technicians studying for the examination in Chemical Pathological Technique of the Institute of Medical Laboratory Technology. As the tests described are also used in many laboratories not directly concerned with diagnosis and treatment, so far as possible the needs of workers in medical research laboratories have also been borne in mind.

While the purpose of the book is essentially practical, it was felt that summaries of the findings in health and disease would add considerably to its value. Although these are necessarily brief they are provided to obviate frequent reference to larger works on the interpretation of biochemical tests. No claim is made that these replace entirely such books a few examples of which are given in the short bibliography on p. 600.

My debts to other authors are many. In a rapidly developing subject such as this much is owed to the various journals which publish papers on biochemical methods. Full references to these are given. Books on practical methods which I have found valuable are also listed in the bibliography already referred to.

I wish gratefully to acknowledge permission to use material by the following: *Acta medica Scandinavica*, for material from Supplement 128 (1942) by Dr. H. O. Lagerlöf; *American Journal of Physiology* for Table XX; Messrs. Baird and Tatlock for Fig. 6; Bayer Products Ltd. for Fig. 4; British Drug Houses Ltd. for the list of primary standards on p. 593; Messrs. J. and A. Churchill Ltd. for normal values for hæmoglobin from "Practical Hæmatology," by J. V. Dacie on p. 456; Evans Electroselenium for Figs. 62 and 63; Harvard University Press for Fig. 64 adapted from "Chemical Anatomy, Physiology and Pathology of Extracellular Fluid." by J. L. Gamble; Ilford Ltd. for Figs. 10 and 11; *The Journal of Clinical Endocrinology* and Dr. S. Soskin for Fig. 14; *The Lancet* for Figs. 18, 19 and 40; *The Lancet* and Dr. J. D. Robertson for Table XXI; Messrs. H. K. Lewis and Co. Ltd. for material on p. 284 from the *British Journal of Experimental Pathology;* Messrs. E. S. Livingstone for the table of Standard Weights on p. 598 from "A Textbook of Dietetics," by L. S. P. Davidson and I. A. Anderson; Dr. G. Lusk for Table XXIII; The Josiah Macy Jr. Foundation for material from the booklet "Copper Sulphate Method for Measuring Specific Gravities of Whole Blood and Plasma," by Phillips *et al.*;

Messrs. May and Baker Ltd. for material on pp. 579–582 from their booklet "The Estimation of Sulphonamides"; Dr. M. Somogyi for Table XVI; Dr. J. H. van de Kamer for Fig. 60; Drs. D. D. van Slyke and G. E. Cullen for Table XIX; Messrs. Williams and Wilkins for material on Indicators and Buffers on pp. 594–596 from "The Determination of Hydrogen Ions," by W. M. Clark (published in Great Britain by Baillière, Tindall and Cox Ltd.); Year Book Publishers, Chicago, for Table XXIX from "Methods in Medical Research," Vol. 2, edited by J. H. Comroe.

I should like especially to express my thanks to my wife who did nearly all the diagrams and formulæ, and to several of my friends and colleagues from whose help I have benefited considerably. Dr. J. E. Kench has read the whole of the proofs whilst Dr. H. T. Howat, Dr. S. W. Stanbury, Mr. M. Bell and Mr. J. E. Southall have each read sections. I have received constant encouragement and advice from Dr. R. W. Fairbrother. For any errors either of omission or commission which still remain the author must accept sole responsibility.

Finally, I should like to thank my publishers for their unfailing consideration and patience during the period in which the book was being written and published.

H. V.

October, 1953

PREFACE TO THE SECOND EDITION

ALTHOUGH little more than three years had elapsed since this book was published when the first edition was exhausted it was clear that considerable revision would be required before it could be reissued. As a result the chapter on Hormones has been largely rewritten and extensive alterations made to the chapter on Proteins. New sections on Hydroxyindoles, Transaminases and Mucoproteins have been added and many new techniques for determining substances in the body fluids have been introduced. Furthermore, numerous minor alterations and corrections have been made. Although a certain amount of material has been deleted, the book is longer by some eighty pages, and it is clear that for any future edition more drastic pruning and rewriting will be required.

I am pleased to acknowledge permission to use material from the following: *The Biochemical Journal* and Dr. J. D. Acland and Mr. P. M. G. Broughton for Figs. 61 and 77; "Clinical Chemistry" for Fig. 74; "Clinica Chimica Acta" for Fig. 38; Evans Electroselenium for Fig. 44; *The Journal of Clinical Investigation* for Fig. 39; Shandon Scientific Company for Figs. 36, 37, 41 and 42.

Once again I particularly wish to thank my wife for doing the

additional diagrams and formulæ, and those of my friends and colleagues who have read, criticized and corrected portions of the book in proof. In this respect my thanks are due especially to Mr. M. Bell and Miss E. M. Hammond, and to Dr. J. E. Kench, Dr. H. T. Howat, Dr. H. Lempert and Dr. D. Longson. I am also indebted to Mr. M. Bell for allowing me to use unpublished results in connection with catecholamines. I am also grateful to the many persons who have written to me, both for the encouragement they have given me and for pointing out errors, which it is my hope will now be very few in number. For such as may still remain I must once again accept sole responsibility.

H. V.

August, 1958.

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CHAPTER I

INTRODUCTORY. COLLECTION OF SPECIMENS AND SOME GENERAL TECHNIQUES

BLOOD

Collection of Blood Specimens

CAPILLARY or venous blood is used for almost all determinations made on blood.

Capillary blood is most frequently obtained from a finger or thumb. The most convenient place is on the thumb about half a centimetre from the side of the nail. The tip of a finger is also used. The finger or thumb is first cleaned with ether or methylated spirit, and is then pricked smartly with a sharp surgical needle. A better flow of blood is obtained when the hand is warm, and if the arm is first allowed to hang limply and is then gently swung backwards and forwards for a short time. After making the prick, a tourniquet of thin narrow tubing is wrapped round from the base of the thumb outwards, and sufficient pressure exerted to force out the blood, which is allowed to run directly into a suitable pipette held inclined at an angle slightly downwards from the horizontal. When the technician is familiar with the technique, the pipette, in many cases, fills easily and quickly. If, however, blood ceases to flow before the calibration on the pipette is reached, it is better not to exert undue pressure, but to unwrap the tourniquet and allow the arm to swing loosely for a moment or two to restore the circulation of the thumb. The tourniquet is then rewrapped and more blood collected. From two to three minutes is available before the blood begins to clot.

Special attention should be paid to ensuring that the pipettes used are clean and dry, otherwise they will not fill easily. Pipettes are cleaned in chromic acid, washed free from acid with water, and dried by drawing a small quantity of acetone through by means of a suction pump. To complete the evaporation of the acetone, the pipettes can be warmed slightly by passing them quickly through a Bunsen flame. A pipette with a straight bore (Fig. 1) is more satisfactory than the type with a bulb. When using the latter, a small bubble of air sometimes remains in the bulb when the blood enters the neck of the pipette.

This method is usually employed when it is desired to collect 0·1 or 0·2 ml. The blood is then placed immediately into some other solution so that no anticoagulant is required. In the hands of a skilled technician as much as 0·5 to 1·0 ml. can sometimes be obtained. In this case the blood can be allowed to drop into a small tube containing a little anticoagulant.

Some workers prefer to use the lobe of an ear. The technique is rather more difficult to acquire. Again amounts up to 0·2 ml. are collected in a pipette, larger volumes into a small tube containing anticoagulant. Amounts up to 3 ml. can be obtained by allowing blood to accumulate repeatedly and then expressing into such a tube. This is particularly valuable when it is difficult to obtain venous blood. In the case of infants the heel is used. The ankle is grasped to congest the foot and a prick made in the heel.

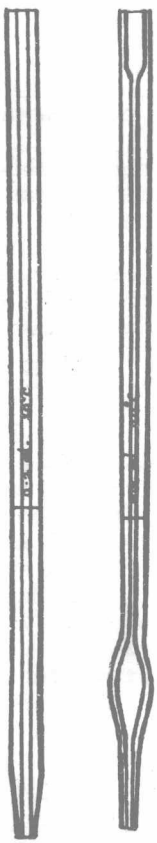

FIG. 1. Blood pipettes.

These methods for collecting blood are widely used. Several ordinary micromethods have been adapted to use 0·1 or 0·2 ml. of capillary blood thus avoiding the use of venipuncture for these determinations.

Capillary blood is similar in composition to arterial blood.

Venous blood must be collected for a large number of estimations. If serum is required the blood is allowed to clot, but is mixed with an anticoagulant if whole blood or plasma is to be used. While the blood may be taken from any prominent vein, a vein on the front of the elbow or forearm is almost universally employed. The arm should be warm. This improves the circulation and distends the vein. The surface of the skin is cleaned by rubbing with alcohol or ether. The arm is extended and a rubber tourniquet firmly applied a few inches above the elbow. This should not obliterate the arterial pulse at the wrist. A well sharpened sterile hypodermic needle fixed on to a syringe of appropriate capacity—usually 10 ml., though if a number of tests are to be carried out on a single specimen, a 20 ml. syringe may be required—is inserted into the vein, which can be held steady by the thumb of the other hand of the operator. When the needle enters the vein the plunger is withdrawn slightly. If blood appears the tourniquet is released, since as little congestion as possible should be present. When the desired amount of blood has been drawn into the syringe, a small pad of cotton wool soaked with spirit or ether, is placed on the arm where the needle was inserted, and the needle withdrawn. This pad is held on for a few minutes until bleeding stops. The needle is removed from the syringe and the blood transferred to an appropriate container, using the minimum amount of pressure. Immediately after use the needle and syringe are washed out with cold water to remove any blood remaining.

Arterial blood is rarely examined. It has been taken for blood gas determinations and when studying the arterial-venous ratio for the blood sugar. It is most commonly obtained by inserting a needle into the radial, brachial, or femoral artery, usually under a local anæsthetic.

Cleaning and Sterilizing Syringes

The piston is removed and the barrel washed thoroughly with water, using a test tube brush. The piston is also rubbed with the brush, special care being taken to see that the back of the piston ring is kept clean. To dry the syringe a little methylated spirit or acetone is drawn in and then expelled as completely as possible. The piston after drying is smeared with a little liquid paraffin as lubricant, then replaced in the barrel and the needle attached. The whole is then placed in a container, the mouth of which is well plugged with cotton wool to prevent movement of the syringe. A glass test tube can be used, in which case the needle is covered with a small metal or glass tube to protect the sharp end, but a metal container is better. Hospital Appliances Ltd., Finchley Wood, Surrey, supply shaped aluminium ones which are very satisfactory (see Fig. 2). If these are used, after plugging with cotton wool, a push fit cap is fitted and the outfit sterilized in an autoclave or dry air oven. A viscose ring stamped STERILE IF LABEL UNBROKEN is placed over the junction of the cap and the container so that the cap cannot be pulled off without breaking it. This method of sterilizing can only be used for all-glass syringes, or for record type syringes with ceramic pistons. If syringes have metal pistons the barrel and piston should be disconnected and sterilized separately.

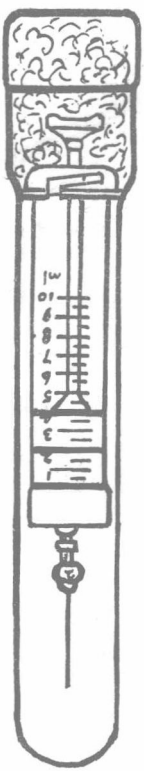

FIG. 2. Container for sterilizing Syringes.

All types of syringe can be sterilized by means of hot oil from an oil bath. The hot oil (liquid paraffin is suitable) at 130° C. to 140° C. is drawn into and forced out of the syringe several times over a period of a half to one minute. This method has been widely used in the past, but has been criticized on the grounds that sterilization may not be effected. This may be so if the oil is not kept long enough in the syringe, as may be the case when it is only drawn in once or twice and is quickly ejected.

Sterilization by placing the parts of the syringe in boiling water (containing a pinch of sodium bicarbonate to prevent rusting) for twenty minutes, has been used, but is not recommended. A little water left in the syringe may cause some hæmolysis.

Sharpening Needles

Efficient sharpening of the needle is most important. A white Arkansas stone (Fig. 3), such as is used by joiners for sharpening gouges, has been found satisfactory. The stone has two edges with different bevels. Needles are sharpened on these edges, which have curves suitable for those of the cutting edges of different sizes of needles. The use of xylol as a lubricant will prevent glazing of the surface of the stone. The needle, held firmly near to the point by the thumb and first finger, is drawn backwards and forwards a distance of 2 to 3 cm. along the edge chosen, and never allowed to leave the surface of the edge while this is being done. When the sharpening is finished the slightly rough outer edges of the bevel can be smoothed by gently rubbing them on the flat surface of the stone.

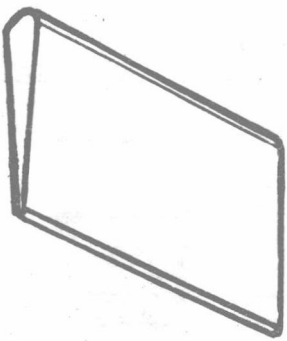

FIG. 3. Stone for sharpening Needles.

Venules

For use outside hospitals, and for those who only wish to take a specimen of blood occasionally, venules (Fig. 4) are convenient. The sterile needle is supplied covered with a small glass tube, which is broken by cutting with the small file included, and the needle then inserted into a vein in the usual way. By manipulating the tube

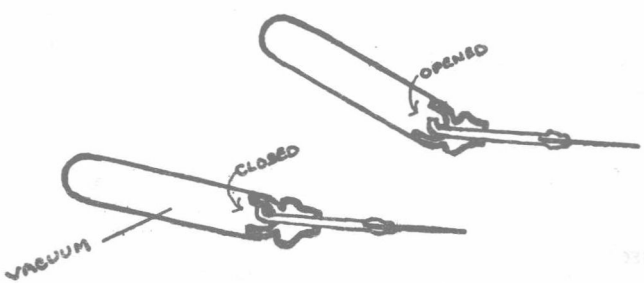

FIG. 4. Venules (Bayer Products, Ltd.).

as shown in the diagram, the valve in the rubber stopper is opened, and the blood is drawn into the tube by the negative pressure inside. The blood is mixed with the anticoagulant by gentle shaking or by rotating the tube between the palms of the hands. Venules can be obtained, either with or without anticoagulant, for a variety of purposes from Bayer Products Ltd., London. Full instructions are given with the tubes.

Avoiding Hæmolysis

When plasma or serum is to be used it is essential to avoid hæmolysis. Even when hæmolysis is not noticeable on inspection, in many specimens spectroscopic examination will show the presence of bands of oxyhæmoglobin. Provided these are faint, their presence may be ignored, but any appreciable degree of hæmolysis, certainly visible hæmolysis, makes the serum or plasma unsuitable for some determinations. It is easier to obtain serum almost free from hæmolysis than plasma, since the anticoagulant present is an additional factor which may produce lysis of the cells.

Certain precautions can be taken to avoid hæmolysis. The syringe and needle must be dry. As lubricant a little liquid paraffin may be used if desired. When taking blood, a minimum amount of constriction is applied to the arm. The blood should flow slowly and steadily into the syringe, and after removing the needle, should be expelled slowly into the tube used. Mechanical breakdown of red cells is thus avoided as far as possible. Tubes into which the blood is placed should be clean and dry. The anticoagulant should be mixed with the blood by gentle rotation. Excessive amounts of anticoagulant should not be used. Separation of plasma should be done by centrifuging at low to moderate speeds. If the plasma is not required immediately, the cells can be allowed to sediment out, the supernatant fluid removed and finally cleared of any remaining cells by centrifuging. In the case of serum, the blood may be allowed to clot in a centrifuge tube, cooled for some time in the refrigerator, and then centrifuged; or the blood is allowed to stand until the clot has contracted, when the serum can be poured off, and centrifuged. If it is necessary to loosen the clot, this should be done as gently as possible with a thin, clean, dry glass rod. Another useful practice is to put the blood into a wider tube than usual and allow clotting to take place with the tube slanted. When the blood is firmly clotted, the tube is stood upright so that the serum then drains off.

Anticoagulants

Heparin is the most satisfactory anticoagulant since it produces no change in the blood. Nevertheless, because they are much cheaper and more easily obtained, oxalates and citrate have been most used. It is important not to use more of these salts than is necessary, otherwise appreciable changes in the distribution of water between cells and plasma may result, and they may interfere with some determinations.

Potassium or Sodium Oxalate. These act by precipitating the calcium. Potassium oxalate has been most commonly used of the oxalates since it is the most soluble. Ten to 20 mg. per 10 ml. blood are required to prevent clotting, but 20 to 30 mg. should be used. Technicians making up tubes should become familiar with this amount. The salt is used finely powdered. Peters and Van Slyke (1932) recommend preparing a 30 per cent. solution of neutral

potassium oxalate, recrystallized if necessary, and adjusted to a *p*H of 7·4 ± 0·2 by adding potassium hydroxide or oxalic acid solution. An appropriate amount, 0·1 ml. for 10 ml. of blood, is measured into the tube which is to hold the blood, is spread on the walls as a film by rotating the tube, and then dried in a stream of air. The blood then mixes easily and rapidly with the oxalate and very little further shaking is necessary. This is specially useful for specimens to be taken under oil.

Sodium Citrate. Citrate does not precipitate the calcium but converts it into a non-ionized form. Nevertheless citrated plasma is not as satisfactory as serum for the determination of calcium. About 30 mg. of sodium citrate per 10 ml. of blood will prevent clotting. About twice this amount should be used.

Ammonium Oxalate (3 parts) + Potassium Oxalate (2 parts) has been used (Heller and Paul, 1934) in certain hæmatological investigations since it does not affect the red cell volume. One mg. per ml. has been suggested, but 2 mg. per ml. may be necessary to ensure that clotting does not occur. Ammonium oxalate should not be included in the anticoagulant for any determination in which ammonia is being produced, for example, in urease urea estimations, Kjeldahl protein and non-protein nitrogen determinations.

Heparin is effective when about 2 mg. per 10 ml. of blood is used. It should be used when it is important to ensure that no change takes place in the blood.

Sodium Fluoride also acts as an anticoagulant, but larger amounts are required than of either citrate or oxalate, so that it is chiefly used as a preservative, when it is usually mixed with potassium oxalate (see p. 7). It is not recommended as an anticoagulant for as much as 100 mg. per 10 ml. of blood is necessary. Fluoride is an enzyme poison.

Changes in Blood on Keeping

The following are among the changes which may take place in blood when it is kept.

1. Loss of carbon dioxide. Since the carbon dioxide content of plasma is higher than that of air, some is lost from the plasma to the atmosphere. This leads to the passage of carbon dioxide from the cells into the plasma, formation of carbon dioxide from bicarbonate in the cells, and to compensate for this, passage of bicarbonate from plasma to cells. Even so, the bicarbonate content of the cells is reduced so that chloride passes from cells to plasma to keep the ratio of bicarbonate to chloride the same in both cells and plasma. In order to prevent loss of carbon dioxide and this series of changes, the blood is collected under liquid paraffin and the plasma separated immediately.

The simplest procedure is to take the blood into a syringe containing a little liquid paraffin but no air, and transfer the blood immediately to a centrifuge tube containing liquid paraffin and oxalate. The end of the needle is placed under the liquid paraffin in this tube and the blood gently forced out. It is mixed with the anti-

coagulant by rotating the tube between the palms of the hands. The tube is then filled with liquid paraffin, corked and centrifuged. Air should be excluded during centrifuging since carbon dioxide may be lost when the blood is shaken. The plasma should be removed from the cells immediately after centrifuging, and care taken not to include any oil when pipetting from this plasma.

For work involving a high degree of accuracy Peters and Van Slyke (1932) collect the blood over mercury, and describe an apparatus by which this can be done.

2. Conversion of glucose to lactic acid, glycolysis (see p. 32).

3. Increase in plasma inorganic phosphate due to formation from ester phosphate present in the cells (see p. 349). To avoid this, serum or plasma is separated within a short time from taking the blood.

4. Formation of ammonia from nitrogenous substances of which urea is the chief, may occur in blood which has been contaminated with bacteria. This may interfere with the estimation of potassium (see p. 401). The blood should be kept sterile or in a refrigerator.

5. Passage of substances through the red cell envelope. This is important, for example, in the case of potassium which is present in much greater concentration in the cells than in the plasma. Serum or plasma should be separated shortly after taking the blood.

6. Conversion of pyruvate into lactate (see p. 476). The blood should be mixed immediately with the protein precipitant or preserved as described on p. 476.

If blood is to be kept it should be collected under aseptic conditions. As preservative a mixture of potassium oxalate and sodium fluoride in the proportions of three parts to one is used. Sodium fluoride mixed with thymol (20 : 1) has also been employed. John (1926) used 20 mg. of this mixture per 10 ml. of blood but other workers have used more. Roe *et al.* (1927) used up to 100 mg. of sodium fluoride per 10 ml. when the blood was sterile. It could then be kept for several days and still be examined for sugar and non-protein nitrogen. More is required if the blood is not kept sterile. Since sodium fluoride inhibits enzyme activity it interferes with the determination of urea using urease.

In general, serum or plasma should be separated as soon as possible after taking the blood. All blood specimens should be kept in a refrigerator.

Type of Blood to be Used

The reasons for the choice of whole blood, serum or plasma for a determination will be discussed when each substance is being considered. There are, however, some general points of importance.

1. When the substance to be estimated is evenly distributed between cells and plasma, it does not usually matter which is used. It may be more convenient to use whole blood, particularly if the test can be done using 0·2 ml. of blood.

2. There are a number of blood constituents, for example glucose, uric acid, creatinine, in the determination of which, substances

present in the cells behave in the same way as the substance which is being estimated, thus giving higher values than the true ones. Use of serum or plasma avoids interference from these. Whole blood placed into isotonic sodium sulphate has also been used for this purpose (see pp. 32, 35).

3. In some cases variations in plasma level of the substance are significant, for example, for plasma chlorides.

4. Since it is easier to obtain serum free from hæmolysis there is a good case for the use of serum rather than plasma when it is necessary to separate off the cells. Plasma, however, must be used when the separation has to be made immediately. Thus plasma is used in the determination of bicarbonate and chloride. To summarize: use

Whole Blood. Glucose, urea, non-protein nitrogen, sulphonamides.

Serum. Uric acid, creatinine, creatine, calcium, sodium, potassium, cholesterol, bilirubin, acid and alkaline phosphatase, amylase, lipase, albumin and globulin, inorganic phosphorus, carotenes, vitamin A, amino-acids, bromide, thiocyanate, salicylate.

Plasma. Chloride, bicarbonate (alkali reserve), *p*H, fibrinogen, ascorbic acid.

Protein Precipitants

The first stage in the majority of determinations made on blood is to remove proteins. For this purpose many substances are used, chiefly heavy metal salts, acids, and organic substances. Tungstic acid and trichloracetic acid are most often employed.

Tungstic acid (Folin and Wu, 1919). The reagents used are 10 per cent. sodium tungstate (Na_2WO_4, $2H_2O$) and 2/3 N sulphuric acid. The tungstate solution should not be acid to phenolphthalein, or more alkaline than to need 0·4 ml. of N/10 hydrochloric acid to make 10 ml. neutral to this indicator. To prepare 2/3 N sulphuric acid 20 ml. of concentrated acid is diluted to 1 litre, standardized against a known sodium hydroxide solution, and adjusted if necessary. This acid liberates the whole of the tungstic acid from an equal volume of the tungstate, and practically all this is taken down by the protein precipitate giving a filtrate which is just to the acid side of neutral, having a *p*H approximately 6·6.

Folin's technique is to mix 1 volume of whole blood with 7 volumes of water, add 1 volume of tungstate, mix, and then add 1 volume of the sulphuric acid slowly, drop by drop, shaking well during the addition. The mixture is then allowed to stand for ten minutes before filtering. With whole blood the final colour should be dark brown. If this colour is not produced it is necessary to add a little more acid. For this purpose, 10 per cent. sulphuric acid is added one drop at a time, shaking well after each addition. Care should be taken not to add more acid than is required. If, on filtering, the first portion of the filtrate is cloudy, it should be refiltered through the same filter paper. When using plasma or serum, 1 volume is taken with 8 volumes of water and a ½ volume each of tungstate and acid.

Haden (1923) modified this procedure. He mixed 1 volume of

blood with 8 volumes of N/12 sulphuric acid, added 1 volume of the tungstate with constant shaking, allowed to stand for a few minutes, and filtered. Van Slyke and Hawkins (1928) added 1 volume of blood to 9 volumes of a mixture containing one part of 10 per cent. sodium tungstate and eight parts of N/12 sulphuric acid. This is convenient but the mixture does not keep well. It should not be kept for more than a fortnight, and should be discarded if any precipitate forms.

Bacterial action can take place in these filtrates so that estimations should be carried out within a few hours unless a preservative is added. Enzyme action is also possible, so that these filtrates can be used for such methods as the urease method for determining blood urea.

Sodium tungstate has also been used with copper sulphate, the copper tungstate formed acting as the protein precipitant.

Trichloracetic Acid. This gives an acid filtrate, which is valuable when an acid medium is necessary to keep in solution the substance, for example phosphate, which is being estimated. Trichloracetic acid usually gives a greater volume of filtrate from the same amount of blood, and filters more quickly than does the tungstic acid mixture. Ten per cent. trichloracetic acid may be used, 9 volumes being added slowly with constant shaking to 1 volume of blood. Alternatively, one volume of blood can be diluted with 4 volumes of water and 5 volumes of 20 per cent. trichloracetic acid added slowly. Proteins are precipitated completely and do not take any of the non-protein nitrogen with them.

Alkaline Zinc Salts. Somogyi (1930) used two solutions, 10 per cent. zinc sulphate ($ZnSO_4$, $7H_2O$) and 0·5 N sodium hydroxide. Ten ml. of the zinc sulphate, diluted to about 50 ml. with water, should require between 10·8 and 11·2 ml. of the hydroxide to become just alkaline to phenolphthalein. To precipitate the proteins, 1 volume of blood is diluted with 7 volumes of water and 1 volume of zinc sulphate solution added. After mixing, 1 volume of the hydroxide is added slowly with constant shaking. The mixture is allowed to stand for a few minutes and then filtered. As with the tungstate method, if plasma or serum is used, 1 volume is diluted with 8 volumes of water and a half volume of both zinc sulphate and sodium hydroxide added.

Cadmium sulphate has also been used. Somogyi (1945) does not find this any improvement, but has substituted *barium hydroxide* for sodium hydroxide. Whereas zinc sulphate and sodium hydroxide satisfactorily deproteinize whole blood, they may fail to do so completely with plasma. Barium hydroxide and zinc sulphate completely deproteinize whole blood, plasma, and serum. Other advantages claimed for this method are that it does not introduce any salts into the filtrate, and that it precipitates anticoagulants such as oxalate and fluoride, which if present in too large amounts sometimes interfere with the removal of protein. The solutions used are 0·3 N barium hydroxide and 5 per cent. zinc sulphate. An example is given on p. 89 in Somogyi's blood sugar method.

These methods are considered by Somogyi to precipitate along with the proteins, some, if not most, of the material which reduces the solutions used in estimating blood glucose (see p. 32). However, other methods have been used to achieve this. Folin (1930) suggested the use of unlaked blood in order to get a filtrate which did not contain certain non-glucose reducing substances. Since these are mostly present in the cells Folin has added 1 volume of the blood to 8 volumes of a solution containing 15 grams of anhydrous sodium sulphate (or 34 grams of the decahydrate) and 6 grams of sodium tungstate (Na_2WO_4, $2H_2O$) per litre. In this solution the cells are not laked. After standing five minutes, 1 volume of N/3 sulphuric acid is added slowly, whilst mixing gently. The cell envelopes are carried down with the protein precipitate, leaving only the diffusible components in solution. This principle has been used in several later methods, for example by King and his co-workers (see p. 40).

Use of Alcohol, Ether, Acetone. In a few methods, use of these organic substances is convenient, particularly if it is desired to extract some blood constituent into the organic solvent. Thus, for example, alcohol-ether mixtures are used to precipitate proteins and extract fats and cholesterol, whilst alcohol is used in the estimation of bilirubin, and acetone in icteric index measurements.

Measuring Blood

Although ordinary pipettes can be used to measure plasma or serum, they cannot be used with whole blood because of its greater viscosity, without introducing appreciable error. For this reason, pipettes used for measuring whole blood should be calibrated to contain the amount of blood which they are designed to measure, and the blood should be washed out of the pipette. With pipettes measuring small amounts of blood, such as 0·1 or 0·2 ml., the blood is usually delivered into a measured amount of a solution and the pipette washed out by sucking the liquid up into it, and blowing it out, until all the blood is washed out. If the blood can be run into one part of the fluid and another part be used for washing out the pipette before the whole is mixed, it is even less likely that a small amount of blood will be left behind in the pipette.

Small blood pipettes should be calibrated by weighing the amount of mercury they can contain. The specific gravity of mercury is 13·57 at 10° C., 13·56 at 14° C., 13·55 at 18° C. Because of the difference in the shape of the meniscus a slightly smaller volume of mercury is weighed, than the volume for which the pipette is being calibrated. This varies with the diameter of the bore at the graduation mark. Examples are: 0·0005 ml. for a bore of 1·0 mm., 0·0015 ml. for 1·5 mm., 0·0025 ml. for 2·0 mm., and 0·0040 ml. for 2·5 mm. A correction curve is given by Peters and Van Slyke (1932). Once the required amount of mercury has been weighed out in a small beaker, it can be used to calibrate a whole series of pipettes by drawing up the mercury or transferring it from one pipette to another. The mercury should be clean and dry.

When using pipettes delivering larger volumes, the blood should