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PRACTICAL HAEMATOLOGY

FOURTH EDITION

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by

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PREFACE TO THE FOURTH EDITION

The five or so years that have passed since the last edition of Practical Haematology was published have witnessed a remarkable increase in our knowledge of the blood and its diseases. New and potentially important facts have been discovered, old problems have been clarified and simple concepts have been shown to be more complex. Haematology as practised in the laboratory has had to keep in step with this new knowledge. An expansion in size of the present edition reflects these changes. Its general arrangement has not been altered but it has been revised extensively and much of the text has been rewritten: new techniques have been introduced, including some involving the use of automation; some methods have been simplified or modified and others omitted altogether. In our revision we have, however, attempted to keep to our original aim, that is to say, we have tried to set out as precisely and simply as we can the whole range of laboratory procedures which are currently undertaken in the investigation of patients suffering from a blood disease. We again considered the possibility of describing in detail the more important abnormalities affecting the nucleated red cells and leucocytes, but we have once more come to the conclusion that for this to be successful coloured plates would be essential, and as this inevitably would make the book considerably more expensive the idea has again been abandoned. We have, however, added several more photomicrographs of notable red-cell abnormalities.

In points of style we now adhere closely to the recent recommendations of the International Union of Pure and Applied Chemistry and to the extension and refinement of the traditional metric system (SI), as recommended by the Royal Society Conference of Editors.

We have been fortunate in obtaining the help of colleagues with respect to certain chapters. Those on the Laboratory Methods Used in the Investigation of Haemolytic Anaemias and on Blood Groups and the Laboratory Aspects of Blood Transfusion have been written in collaboration with Dr. Sheila M. Worlledge, and that on Investigation of the Haemorrhagic Disorders has been written in collaboration with Dr. W. R. Pitney. Drs M. C. Brain and L. Szur have given us much helpful advice, and we are also indebted to Dr. O. W. van Assendelft of Groningen, and to Dr. G. Garratty, Dr. H. Glass,

Dr. J. R. Hobbs, Mr. J. Morgan and Mr. L. H. Wallett for help on specific points. We should also like to acknowledge permission from the Royal College of Physicians of Edinburgh to reproduce two figures in Chapter 8. As before, we are greatly indebted to Mr. W. H. Brackenbury for his care and skill with the photomicrographs new to this edition. Finally, we have as before received every possible help from the Publishers.

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Chapter 1

COLLECTION OF BLOOD AND NORMAL VALUES

COLLECTION OF BLOOD FROM PATIENTS

VENOUS blood is preferred for most haematological examinations, but peripheral samples can be almost as satisfactory for some purposes if a free flow of blood is obtained.

VENOUS BLOOD

This is best withdrawn from an antecubital vein by means of a dry glass or plastic syringe. Alternatively, a needle to which about 5 cm of plastic tubing have been attached can be used in patients who have good veins. The needles should not be too fine or too long. Those of 19 or 20 SWG* are suitable for use with syringes. If a syringe is not being used, they should be of larger bore, e.g., 16 SWG*; with these needles 100 ml or more of blood can be easily withdrawn. Short needles with shafts about 15 mm long are particularly suitable for use with children.

Except in the case of very young children it should be possible with practice to obtain venous blood even from patients with difficult veins. Successful venepuncture may be facilitated by keeping the subject's arm warm, applying to the upper arm a sphygmomanometer cuff kept at approximately diastolic pressure and smacking the skin over the site of the vein. In obese patients it may be easier to use a vein on the dorsum of the hand, which is warmed by immersion in warm water. When the hand is dried and the fist clenched, veins suitable for puncture will usually become apparent. If the veins are very small, a 23 SWG* needle should be used and this should enable at least 2 ml of blood to be obtained satisfactorily. Vein punctures in the dorsum of the hand tend to bleed more readily than at other sites. The arm should be elevated after withdrawal of the needle and

* The nearest equivalent American gauges and diameters are as follows: 16 SWG = 14 (1·625 mm); 19 SWG = 18 (1·016 mm); 20 SWG = 19 (0·914 mm); 23 SWG = 22 (0·610 mm).

pressure should then be applied for several minutes before an adhesive dressing is placed over the puncture site.

Ideally, congestion should be completely avoided so as to prevent haemoconcentration, although stasis for a period of less than 1 minute has little effect (20a). In practice, it is usually necessary to use a tourniquet. Ideally, this should be loosened once the needle has been inserted into the vein. The piston of the syringe should be withdrawn slowly and no attempt made to withdraw blood faster than the vein is filling. After detaching the needle, the blood should be delivered carefully from the syringe into a container, and if it is desired to prevent coagulation it should be promptly and thoroughly, but gently, mixed with the anticoagulant.

Ideally, blood films should be made immediately the blood has been withdrawn. It is convenient to deliver a small volume of blood on to a glass slide and to take from this blood small sub-samples sufficient for a single film, using a short length of glass capillary tubing into which the blood will run by capillarity. In practice, blood samples are often collected by the clinical staff and sent to the laboratory after a variable delay. Films should be made in the laboratory from such blood as soon as is practicable. Again, a glass capillary can be used to sample the blood and to deliver a drop of the right size on to a slide so that a film can be made.

The differences between films made of fresh blood (no anticoagulant) and anticoagulated blood are dealt with on p. 6.

It is convenient to use as containers for blood samples disposable plastic flat-bottomed tubes fitted with plastic stoppers, glass or plastic tubes with rubber bungs or small glass bottles with rubber-lined screw caps. The requisite amount of anticoagulant should be added and allowed to dry (see p. 5). The most common disposable plastic container available from commercial sources contains dipotassium EDTA as anticoagulant and is marked at the 2·5-ml level to indicate the correct amount of blood to be added.

Haemolysis can be avoided or minimized by using clean apparatus, withdrawing the blood slowly, not using too fine a needle, delivering the blood gently into the receiver and avoiding frothing during the withdrawal of the blood and subsequent mixing with the anticoagulant.

A convenient method for collection of blood, especially when only a single blood sample is required, is by means of an evacuated specimen tube and needle* which obviates the need for a syringe.

* e.g., "Vacutainer" (Becton, Dickinson and Co., 1 North Court, London, S.W. 1.).

Serum

Blood collected in order to obtain serum should be delivered into sterile tubes or screw-capped bottles and allowed to clot undisturbed for 1–2 hours at 37°C. When the blood has firmly clotted and the clot has started to retract, the sample may be left in a refrigerator overnight at 4°C, so that clot retraction may become complete under conditions unfavourable for the growth of bacteria. If the clot fails to retract, it may be gently detached from the walls of the container by means of a platinum wire or sealed Pasteur pipette. If it is roughly treated, haemolysis is certain to follow. However, exactly how serum should be obtained depends also on what it is required for. For instance, if complement is to be estimated, the serum should be separated and then frozen at −20°C or below with the minimum of delay.

When serum is required with the minimum of delay or when both serum and cells are required, as in the investigation of certain types of haemolytic anaemia, the sample can be defibrinated. This is simply performed by placing the blood in a receiver such as a conical flask containing a central glass rod on to which small pieces of glass capillary have been fused (Fig. 1.1). The blood is whisked around the central rod by moderately rapid rotation of the flask. Coagulation is usually complete within 5 minutes, most of the fibrin collecting upon the central rod. When fibrin formation seems complete, the defibrinated blood may be centrifuged and serum obtained quickly and in relatively large volumes. Blood defibrinated in this way should not undergo any visible degree of haemolysis. The morphology of the red cells and the leucocytes is well preserved. Defibrinated blood is thus a good source of leucocytes for buffy-coat preparations.

If cold agglutinins are to be titrated, the blood must be kept at 37°C until the serum has separated and if cold agglutinins are known to be present in high concentrations it is best to bring the patient to the laboratory and, using a needle connected to 7–8 cm of plastic tubing, to collect blood into containers previously warmed to 37°C. When filled they should be promptly replaced in the 37°C water-bath. In this way it is possible to obtain serum free from haemolysis even when cold antibodies are present capable of causing agglutination at temperatures as high as 30°C. Alternatively, the blood may be collected in a warmed syringe. A practical way of achieving this is to place the syringe in its container for 10 minutes in an oven at approximately 50°C. When the clot has retracted and clear serum has been expressed, the serum is removed by a Pasteur pipette and transferred to a tube which has been warmed by being allowed to stand in the water-bath. It is then rapidly centrifuged so as to rid it of any suspended red cells.

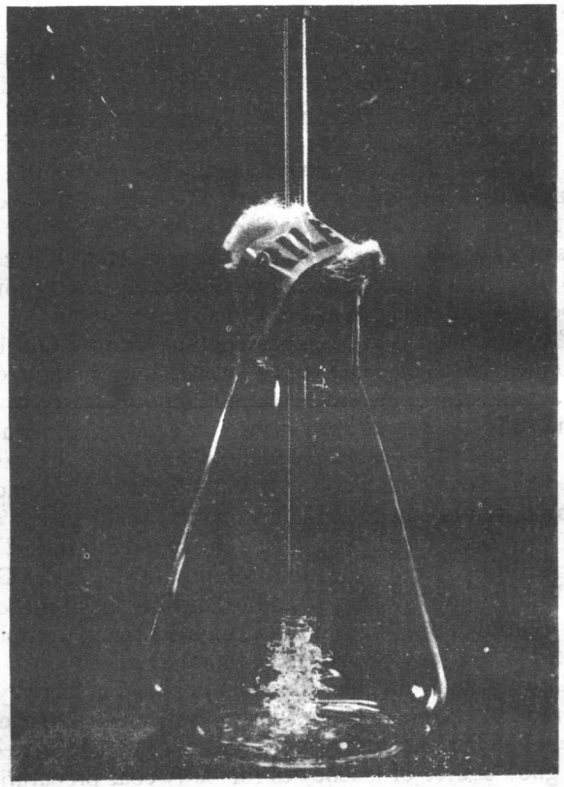

FIG. 1.1. FLASK FOR DEFIBRINATING 10–50 ml OF BLOOD
The central glass rod has had some small pieces of drawn-out capillary fused to its lower end.

ANTICOAGULANTS

Several good anticoagulants are available for use in clinical haematology: disodium or dipotassium ethylenediamine tetra-acetic acid (EDTA; Sequestrene); ammonium and potassium oxalate mixture; sodium citrate, and heparin.

EDTA

The disodium or dipotassium salts of EDTA are powerful anticoagulants which are more effective weight for weight than is sodium citrate (10, 11). As they have definite advantages over potassium and ammonium oxalate mixture they have largely replaced the latter as the anticoagulant of choice in routine haematological work. They

are effective at a concentration of 1 mg/ml of blood. EDTA in excess of 2 mg/ml of blood may result in a significant decrease in the packed cell volume (15). Care must be taken to ensure that the correct amount of blood is added, and that by repeated inversions of the container the anticoagulant is thoroughly mixed in the blood added to it. The dipotassium salt is more soluble than the sodium salt and is to be preferred on this account.

The dilithium salt of EDTA is equally effective as an anticoagulant (28), and its use has the advantage that the blood can then also be used for chemical investigations. However, it is less soluble than the other salts and it is more difficult to prepare a stock solution for dispensing into individual containers.

Ammonium and Potassium Oxalate Mixture

The mixed salts (six parts of ammonium oxalate to four parts of potassium oxalate) are used at a concentration of 2 mg/ml of blood. The mean corpuscular volume is unaltered and little haemolysis caused. Such blood may be used for packed cell volume (PCV) and haemoglobin estimation, and for red-cell and leucocyte counts, and the plasma used for the estimation of bilirubin.

Trisodium Citrate

0·106 mol trisodium citrate (31·3 g/l. $Na_3C_6H_5O_7.2H_2O$)* is the anticoagulant of choice in coagulation studies. Nine parts of blood are added to one part of the sodium citrate solution and immediately well mixed with it. Sodium citrate is also the anticoagulant most widely used in the estimation of the sedimentation rate (ESR); four parts of venous blood are diluted with one part of the sodium citrate solution.

Heparin

This may be used at a concentration of 10–50 int. units per ml of blood. Heparin is an effective anticoagulant and does not alter the size of the red cells; it is a good dry anticoagulant when it is important to reduce to a minimum the chance of haemolysis occurring after blood has been withdrawn. However, heparinized blood should not be used for making blood films as it gives a faint blue coloration of the background when the films are stained by Romanowsky dyes. This is especially marked in the presence of abnormal proteins. Heparin is the best anticoagulant to use for osmotic fragility tests; otherwise it is inferior to EDTA or mixed oxalates for general laboratory use.

* Or 38 g/l. $2Na_3C_6H_5O_7.11H_2O$. This salt is, however, less readily obtained.

Comparison between EDTA and Oxalates

The main advantage that salts of EDTA have over potassium and ammonium oxalate mixture is that they most effectively prevent the clumping of platelets *in vitro*. Their use, therefore, not only permits platelet counts to be carried out on venous blood samples sent to the laboratory but they also enable rough estimates of platelet numbers to be made by inspection of a stained film. When other anticoagulants are used—in which platelets clump rapidly—any such estimates are unreliable.

There seems little to choose between mixed oxalates and EDTA from the point of view of the degenerative changes which take place in the blood cells when the blood is allowed to stand. Films from blood containing EDTA should therefore be made as soon as possible after the blood is collected. EDTA is not a suitable anticoagulant for use in the investigation of coagulation problems and should not be used in the estimation of prothrombin time.

Effects of Anticoagulants on Blood-Cell Morphology

It is worth recording the degenerative changes which affect leucocytes when blood is allowed to stand in the laboratory before films are made. The changes are not wholly due to the presence of an anticoagulant for they occur, although considerably more slowly, in defibrinated blood; however, in the presence of dipotassium EDTA they are less marked than in blood containing ammonium and potassium oxalates. The changes appear to be proportional to the concentration of anticoagulant.

Irrespective of anticoagulant, films made from blood which has been standing for not more than 1 hour at 18–20°C are not easily distinguished from films made immediately after collecting the blood. At 3 hours, however, the changes are quite well marked, especially in oxalated blood. Some but not all the neutrophils will be affected: the nuclei stain more homogeneously than in fresh blood and the nuclear lobes may become separated and the cytoplasmic margin may appear ragged; small vacuoles appear in the cytoplasm. Some of the large mononuclears develop extensive changes. Small vacuoles appear in the cytoplasm and the nucleus undergoes irregular lobulation which may almost amount to disintegration (Fig. 1.2). A minority of the lymphocytes undergo a similar type of change: small vacuoles develop in the cytoplasm and the nucleus may undergo budding so as to give rise to nuclei with two or three lobes (Fig. 1.3). Other lymphocyte nuclei stain more homogeneously than usual.

At 6 hours all these changes are marked, but are more severe with the mixed oxalates than with EDTA. The leucocytes in defibrinated blood, on the other hand, will be found to be hardly affected. The red cells

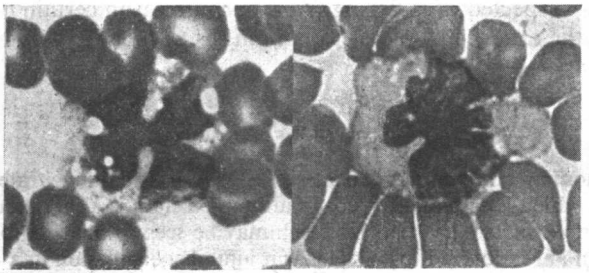

FIG. 1.2. EFFECT OF AN ANTICOAGULANT ON LEUCOCYTE MORPHOLOGY
Photomicrographs of monocytes in a film made from EDTA-blood after 18 hours at 20°C. ×1200.

(of normal blood at least) are little affected by standing for 6 hours at room temperature. Longer periods, however, lead to progressive crenation and sphering. It is interesting to note that it does not seem possible to delay the onset of the progressive changes which affect both the leucocytes and red cells by keeping the blood cool at 4°C.

The occurrence of the above-described changes underlines the importance of making films of blood as soon as possible after withdrawal. But delay of up to an hour or so is certainly permissible.

The practice of making films of blood before it is added to the anticoagulant (e.g., at the bedside) is to be commended. In such films, however, the platelets normally clump and it is less easy to estimate roughly the platelet count from inspection of the film. Films of fresh blood should always be made in the course of investigating

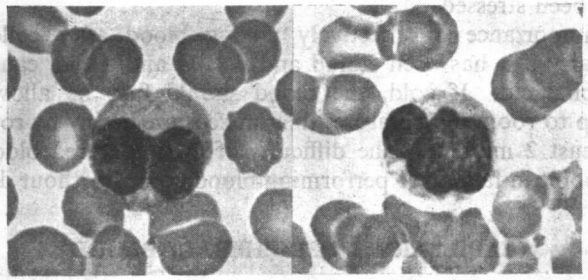

FIG. 1.3. EFFECT OF AN ANTICOAGULANT ON LEUCOCYTE MORPHOLOGY
Photomicrographs of lymphocytes in a film made from EDTA-blood after 18 hours at 20°C. ×1200.

patients suspected of suffering from purpura, as in certain rare conditions the absence of platelet clumping is a valuable pointer to the diagnosis (see p. 319).

Mode of Action of Anticoagulants

Ammonium and potassium oxalates, EDTA and sodium citrate remove calcium which is essential for coagulation. Calcium is either precipitated as insoluble oxalate (crystals of which may be seen in oxalated blood) or bound in an un-ionized form. Heparin inhibits coagulation in a different way; it is thought to have the power of neutralizing thrombin in the presence of a co-factor located in the albumin fraction of serum. Ammonium and potassium oxalates are poisonous and are for laboratory use only. Sodium citrate, EDTA or heparin can be used to render blood incoagulable before transfusion.

Storage of Blood before Estimations are Performed

Regardless of the anticoagulant, certain changes take place when blood is allowed to stand *in vitro*. They can be minimized by keeping the samples in a refrigerator at 4°C. The red cells start to swell, with the result that the mean corpuscular volume increases, osmotic fragility and prothrombin time slowly increase and the sedimentation rate decreases; the leucocytes gradually autolyse (7). Haemoglobin remains unchanged for days, provided that the blood does not become infected. However, these changes take place slowly, and for many purposes blood may be safely allowed to stand overnight in a refrigerator if precautions against freezing are taken. Nevertheless, it is best to count leucocytes and platelets and to measure the packed cell volume within 6 hours of collection and to estimate the sedimentation rate within 2 hours. The advisability of making films at once has already been stressed.

The importance of effectively mixing blood after collection, particularly if it has been stored and is cold and viscid, cannot be overemphasized. If cold, the blood should first be allowed to warm up to room temperature, then mixed, preferably by rotation, for at least 2 minutes. The difficulty of mixing stored blood is a strong point in favour of performing blood counts without delay.

"CAPILLARY" (PERIPHERAL) BLOOD

This can be obtained from the ear-lobe or finger of an adult or from the heel of an infant. As already mentioned, a free flow of blood is essential, and only the very gentlest squeezing is permissible; ideally, large drops of blood should exude slowly but spontaneously.

If it is necessary to squeeze firmly in order to obtain blood, the results are unreliable. If the poor flow is due to the part being cold and cyanosed, too high figures for red-cell counts, haemoglobin content and leucocyte counts are usually obtained.

The discrepancies between peripheral and venous samples are more marked if the ear-lobe rather than the finger is chosen as the site for puncture (3, 17). However, if the ear is rubbed well with a square of lint until it is pink and warm, a good spontaneous flow of blood is obtained from most patients if steel lancets or glass capillaries are used as prickers (see below). Under these circumstances the figures for red-cell counts, haemoglobin content and leucocyte counts approximate closely to those of venous blood. In "screening" work the authors prefer to obtain blood from the ear rather than the finger because puncture of the ear-lobe is less painful and a good flow of blood can usually be obtained. Particularly if a glass capillary pricker is used quite large volumes of blood can readily be obtained almost painlessly if the ear is skilfully punctured. Individually wrapped sterile disposable steel lancets are now widely available from commercial suppliers, and for routine purposes have replaced glass-capillary prickers in the authors' laboratory. The making of glass-capillary prickers is described in the previous edition of this book.

Ear-lobe puncture is carried out as follows. The ear must be rubbed with lint until warm. It is then pricked to a depth of 2–3 mm with a sterile steel lancet or glass-capillary pricker. The lancet is thrust into the ear-lobe by a single stabbing action, the glass capillary is placed against the most dependent part of the lobe of the ear and inserted by gentle rotation. The first few drops should be wiped away and the sample collected when the blood is flowing spontaneously, usually in about half a minute. A separate lancet or capillary pricker is used for each patient.

Glass capillaries are too fragile for use in pricking the finger. A sterile steel lancet should be used instead. If a Hagedorn or similar needle is used for pricking the finger or ear, it is essential to use a fresh properly sterilized needle for each patient, for only by flaming the needle between each puncture—which soon results in blunting it—can it be effectively sterilized. Dipping in alcohol is insufficient to exclude the possibility of transmitting serum hepatitis from one patient to the other.

Heel Blood

Satisfactory samples can be obtained in infants by a deep puncture using a stout needle or steel lancet, but only if the heel is really warm—it may be necessary to bathe it in hot water. Even so, skin-prick

samples give haemoglobin values somewhat higher than do venous samples. Particularly is this true of the first day of life. Mollison (20b) stated that the haemoglobin concentration of heel blood is about 5% higher than that of venous blood and mentions that other authors have recorded differences of up to 15%. Oh and Lind (25) have shown that in the first few hours of life the discrepancy is especially marked in infants in whom the cord has been clamped late. These authors give formulae by means of which capillary haematocrit measurements can be converted to venous packed cell volume on the basis of experimentally determined regression lines.

Collection of Capillary Blood for Quantitative Studies (16)

The usual procedure is to use a micropipette to draw up the correct amount of blood (usually 0·02 ml). An alternative method is to use disposable capillary tubes cut to size so as to contain the exact volume of blood when completely filled. The capillary is allowed to fill by capillarity and is then dropped into a tube containing the appropriate amount of diluent solution; with shaking the blood diffuses rapidly into the solution. A potential disadvantage is the presence of contaminating blood on the outside of the capillary where it has been in contact with the flow. Such blood is difficult to wipe off without causing the loss of a portion of the blood contained within the capillary. However, by providing a length of tubing greater than that necessary for the required volume, and a break-off point at the correct length, it is possible to fill the capillary from the non-calibrated end, which is discarded. This method avoids contamination of the exterior of the portion of the tube which is placed in the diluent. Break-off capillary tubes can be manufactured within a tolerance of ±2% of the required volume.* This method demands less technical skill for drawing up the correct amount of blood than the micropipette method, and it is particularly suitable as a bedside or "field" technique.

Differences between "Capillary" and Venous blood

It is not quite clear whether the packed cell volume (PCV; haematocrit value), red-cell count and haemoglobin content of venous blood and capillary blood are the same, even if the latter is freely flowing. Although the results of Price-Jones, Vaughan and Goddard (27) did not reveal any certain differences in normal adults, this may not be always true. Strauss and Burchenal (31), in a survey of 80 patients under treatment for pernicious anaemia, found that their red-cell counts and haemoglobin levels averaged 5% higher in capillary than in venous blood, a difference which was statistically significant. In infants, as already mentioned, even greater differences have been reported to exist.

* Harshaw Chemical Co., Daventry.

It is likely that freely flowing blood obtained by skin puncture is more nearly arteriolar in composition than capillary. Indeed, the PCV, red-cell count and haemoglobin content of true capillary blood are significantly less than those of venous blood (9). This results in the venous PCV being normally significantly greater than the "whole-body" PCV, a difference which is of significance in the calculation of total blood volume from an estimation of plasma or red-cell volume (see p. 360).

The platelet count appears to be higher in venous than in capillary blood—this may, however, be due to adhesion of platelets at the site of the skin puncture. Leucocyte counts are probably identical, but only if the peripheral blood is freely flowing—if the ear is cold, the capillary count may be much higher than the venous count (17). The osmotic fragility of venous blood is significantly greater than that of peripheral blood, due probably to the lowered pH and reduced oxygen tension in the venous sample.

NORMAL VALUES IN HAEMATOLOGY

It is extremely difficult to state the limits of haematological values in health; the observed ranges are considerable, and age and sex and altitude determine important differences. In addition, variation in technique may explain differences between some observers' figures; particularly is this true of such estimations as the counting of platelets and the whole-blood coagulation time. Furthermore, it is difficult to be certain that in any survey of a population for the purpose of obtaining data from which a normal range may be constructed, the "normal" subjects are in fact completely healthy and do not have mild chronic infections or nutritional deficiencies.

The borderline between health and ill-health is indefinite; so it is with haematological values, for the normal and abnormal undoubtedly overlap. For instance, a value well within the recognized "normal" range may be definitely pathological in a particular subject, e.g., a total leucocyte count of 10,000 cells per mm^3 is abnormal for a man whose count usually ranges between 4,000 and 6,000/mm^3.

The data given in Table 1.1 are derived from various sources (14, 19, 22, 24, 29; for other references see the 3rd edition of this book). The figures given are believed to cover at least 95% of healthy subjects except in the case of leucocyte counts where variation between subjects is remarkably great and the ranges given cover probably not more than 90% of healthy persons (2). According to Booth and Hancock (2), more counts are met with above than below the usually accepted normal ranges.