STUDIES ON QUALITY ASSURANCE IN HAEMOCYTOMETRY
(Studies over kwaliteitsbewaking
in de hemocytometrie)

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Ter nagedachtenis aan mijn vader
Aan mijn moeder
Aan Diane

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DRUKKERIJ J.H.PASMANS B.V.,'s GRAVENHAGE

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MAJOR ABBREVIATIONS (Not invariably applicable to Chapter IV)

ACD	=	Acid Citrate Dextrose
BCR	=	Bureau Communautaire de Référence; European Community Bureau of Reference, Brussels
BCSH	=	British Committee for Standardization in Haematology
c.c.	=	counting chamber
cf	=	centrifugal or confer
Ch	=	Chapter
Chct	=	Coulter counter haematocrit
conc	=	concentration(s)
CPD	=	Citrate Phosphate Dextrose
CPDA	=	Citrate Phosphate Dextrose Adenine
CV	=	Coefficient of Variation (in %)
DH	=	Degree of Haemolysis
ECCLS	=	European Committee for Clinical Laboratory Standards
EDTA	=	Ethylene Diamine Tetra Acetate
EQA	=	External Quality Assessment
f	=	(prefix) femto = 10^{-15}
Fig.	=	Figure
haem.	=	haematology or haematological
Hb	=	Haemoglobin (mmol/l)
hc	=	haemocytometer
hcs	=	haemocytometers
Hct	=	Haematocrit (%)
hcy	=	haemocytometry
HiCN	=	cyanhaemiglobin
HSLP	=	High-Standard Laboratory Performance
ICSH	=	International Committee for Standardization in Hematology
IFCC	=	International Federation of Clinical Chemistry
IQC	=	Internal Quality Control
Ly	=	Lymphocyte
m	=	(prefix) milli = 10^{-3}
MCH	=	Mean (red) Cell Haemoglobin (fmol)
MCHC	=	Mean (red) Cell Haemoglobin

		Concentration (mmol/l)
MCV	=	Mean (red) Cell Volume (fl)
Mhct	=	Microhaematocrit (%)
MPV	=	Mean Platelet Volume (fl)
n	=	(prefix) nano = 10^{-9}
n	=	number (of persons or measurements, etc)
NCCLS	=	National Committee for Clinical Laboratory Standards (USA)
p	=	(prefix) pico = 10^{-12}
p	=	probability of a result being due to chance
p.	=	page
PDW	=	Platelet Distribution Width
plt	=	platelets or thrombocytes
PLT	=	Platelet counts (10^9/l)
QA	=	Quality Assurance
QC	=	Quality Control
rbc	=	red blood cells or erythrocytes
RBC	=	red blood cell counts (10^{12}/l)
RDW	=	Red cell Distribution Width (fl)
ref	=	reference
S	=	Section
SD	=	Standard Deviation
u	=	(prefix) micro = 10^{-6}
wbc	=	white blood cells or leucocytes
WBC	=	wbc counts (10^9/l)
WHO	=	World Health Organization

Chapter I

INTRODUCTION AND OBJECTIVES

1. Quality Assurance (QA)

1.1. Objectives of QA

Unreliable laboratory results may have serious consequences for the health of individuals or communities. The main objectives of QA are to provide reliable laboratory data in all health care activities and to ensure comparability of results, particularly in epidemiological investigations, health surveys, environmental monitoring , medical research and other public health activities. In the direct clinical care of patients the objectives are to improve the accuracy of clinical diagnosis and therapeutic monitoring, to reduce health care costs, to provide a basis for the continuing education of physicians and laboratory scientists in scientific methods of investigation, laboratory organization and management, and analytical techniques (1).

1.2 Justification of terminology in QA

The choice of terms should be governed by a knowledge of the underlying principles and objectives and by an understanding of the effects that words can have on people's thoughts and actions. In this thesis we adopt an amended (IFCC) terminology, as recently recommended by members of a WHO working group, including haematologists (1-5), and as adopted by ECCLS in their "proposed standard for quality assurance" (27). They point out that the well-known IFCC term "external quality control" (3) is a misnomer in that it provides retrospective quality assessment rather than control over results of a

particular laboratory. On the other hand the total set of procedures defined by IFCC as "internal quality control" does indeed exert "control" over results released by a laboratory (2,4). "Control" involves internal assessment of quality plus the measures taken within the laboratory to maintain and/or improve quality. Internal quality control and external quality assessment are two, fundamentally different components of a comprehensive quality assurance programme. The three terms are defined as follows (2,4):

- Internal quality control (IQC) is the set of procedures undertaken by staff of a laboratory for continuously assessing work and results in order to decide whether they are reliable enough to be released together with the implementation of measures to maintain and/or improve the quality.

Thus, quality control procedures have an immediate effect and should actually control - as opposed to merely examining - the laboratory's output (2,4).

- External quality assessment (EQA), which term should be used in preference to "External quality control", refers to a system of objective checking laboratory results by means of an external agency. The checking is necessarily retrospective and the comparison of a laboratory's performance on a certain day with that of other laboratories cannot be ascertained until some time later, so this comparison will not have any influence on the test laboratory's consistency but will establish between-laboratory comparability. (2,4,5).

- Quality assurance (QA) comprises the constant practice of IQC, participation in EQA schemes and a further series of monitoring, education and training systems concerned not only with specimen examination but also with

specimen collection, transport and handling and with methods for reporting of results. The term "quality assurance" does not imply that perfectly reliable results can be "assured" at all times, but that a systematic effort is being continually made towards achieving the highest possible quality of performance (2,4).

A large number of organisations other than the IFCC are engaged in defining terminology: BCR, BIPM, ECCLS, ICSH, ISO, IUPAC, NBS, NCCLS, WHO, just to mention a few! This has resulted in a wide variety of definitions for the same concepts and terms, often dependent on the special requirements of the specialty concerned. There is a great need for standardisation in terminology. However, since we cannot possibly contribute to a solution to the confusion of terms, in this thesis we adhere to the IFCC terminology, a glossary of which is compiled in Ref (3).

2. Haemocytometry (Hcy)

2.1 Clinical significance of haemocytometry

Haemocytometry (Blood cell counting and sizing) has been an established diagnostic tool of paramount clinical importance for many decades. Since modern clinical haem. textbooks (6,7) extensively deal with the clinical significance of hcy we shall, for the sake of brevity, refrain from reviewing the more common aspects of the subject. Consequently, we shall only briefly highlight here some features of recent clinical progress.

Modern therapeutic treatments add to the ever growing demand by clinicians for an expanded range of blood cell tests of superior precision and accuracy (8,9). This applies especially to the low ranges of both wbc and plt counts where therapeutic decisions are based on relatively small changes of these

quantifications (e.g. transfusions, cessation or resumption of chemotherapy, radiotherapy etc.) (8,9). Reviews on the great clinical importance of low (10-12) and high MCV (12) have recently been updated. An improved classification of anaemias based on MCV, RDW and rbc-histograms has recently been proposed (13-15). Studies on the significance of plt characteristics have only recently begun to appear in the literature (16-22).

Hcy is also of great importance in a quantitative sense. Some 100.000 comprehensive (eight-parameter) blood cell determinations per year are performed in the haem. departments of our 860-bed hospital. It has been estimated that over 10 million full blood counts are carried out in the world each day (23).

Other aspects of the clinical significance of hcy will be dealt with in Ch II, S 4.2. and in Ch III.

2.2. Progress in flow haemocytometry.

The advent of advanced multiparameter instruments for high-speed analyses of blood cells in a fluid suspension (flow haemocytometry) has revolutionized the operation of the modern haem. laboratory. Breakthrough improvements such as hydrodynamic focusing, pulse editing and sweep flow application (24) (Ch III) enable the simultaneous enumeration and characterization of wbc, rbc and plt and contribute to better quality of the measurements of cells and their characteristics. While increasing the operational simplicity these major fluidic and electronic improvements have yielded so-called eight-parameter instruments of improved performance (9). They provide excellent resolution, greater precision and linearity, less carry-over, faster analysis rates and better cost efficiency than the now obsolescent older-generation, automated

haem. analyzers (9). Additionally, these instruments have the potential to provide increased information on hitherto unexploited cellular parameters. They eventually may prove of relevant or even vital clinical importance in the diagnosis, evaluation, treatment and therapeutic monitoring of haem. diseases (15-22). Furthermore, the implementation of these instruments enable laboratories to cope with the ever growing clinical demands for quick and better-quality blood cell analyses over the entire, wide range of haem. values (8,9) (Ch III).

3. Problems of haemocytometry: QA

The progress in instrumentation described above forms the basis for future perfection. However, advancement in instrumentation has exceeded that of QA (24,25). In fact, QA techniques should be brought to the same high level as the instrumentation. The poor state of the art of QA can be attributed to the following facts:

a. Calibration techniques have not kept pace with the improvement in precision (25). Despite the joint efforts of many international bodies (26) "definitive" and ref preparations and methods do not (yet) exist in haem. with the exception of the Hb-determination (26) (Ch II).

b. Insufficient knowledge of and/or attention to the influence of numerous other conditions on haem. values :e.g. sampling, transport, storage, interpretation errors, physiological and pharmacological influences (Ch II).

c. Incompatibility between results of instruments based on different principles (23) (Ch III).

d. The almost complete absence of literature on the preparation of medium-term

multiparameter internal quality control (IQC) materials, let alone standards (Ch IV).

4. Outline and objectives of this thesis

The objectives of this thesis are:

a. to review the state of the art, the concepts, the problems and the perspectives of comprehensive QA in hcy (Chapters II and III);

b. to study the basic principles and problems of cell counting and sizing and the recent progress in approaching these problems; to describe the fluidic and electronic improvements in modern instruments, enabling the simultaneous measurement of wbc, rbc and plt and their characteristics, with special reference to the Coulter Counter Model S Plus-II (Ch III);

c. to develop IQC materials of medium and/or long-term stability with special emphasis on ease of preparation and low costs, thus contributing to optimization of IQC (Ch IV).

Chapter II

STATE OF THE ART OF QUALITY ASSURANCE (QA) IN HAEMOCYTOMETRY

1. Introduction

> "There is no simple over-all answer, and any answer that we find will be temporary, depending on the progress of medical knowledge and technology"
>
> R.N. Barnett 1968 (1)

Haemocytometric testing has many important functions. It generally provides essential data in the processes of diagnosis, prognosis, disease and drug monitoring as well as delineation of risk factors.

In order to assure High-Standard Laboratory Performance (HSLP) (2), Quality Assurance (QA) (3) should be an integral part of the activities of the haematological (haem.) laboratory. Achieving and maintaining HSLP in haemocytometry (hcy) is a challenging task, further compounded by the need to provide the test results quickly, cheaply, and at anytime day or night, including weekends and holidays. Simply stated, HSLP requires obtaining an adequate specimen which is handled properly, analyzed reliably and interpreted correctly (2,4). HSLP should apply to :

a. the pre-analytical phase
   - requisition of the proper test(s)
   - correct preparation of the patient and appropriate drawing, collection, administration, transport and storage of the blood specimen

b. the analytical phase
   - careful monitoring and checking of precision and accuracy of the measuring instrument(s) in internal (IQC) and

external (EQA) programmes

c. the post-analytical phase
   - appropriate recording and reporting of the results
   - appropriate interpretation, follow-up and decision making

Besides, HSLP should encompass favourable cost-benefit conditions, an important aspect in these days of consumerism and governmental budgetary measures in the medical discipline.

In this Chapter we shall elaborate on most of these different aspects of comprehensive QA in hcy with special emphasis on the analytical problems.

2. Pre-analytical phase

Although somewhat taken for granted, the blood specimen is the centre piece of all our efforts, since, quite obviously, the best analysis is worthless if done on an improper specimen. HSLP in hcy requires constant vigilance in order to assure the integrity of the specimen. In many instances we tend to become so absorbed in the analytical process that we gloss over the fundamental principles that deal with obtaining an adequate specimen (2,5,6). These principles comprise the appreciation and assessment of the sources of error inherent in the requisition of tests, the preparation of the patient and the drawing and processing of the specimen before the actual analysis. Consequently, it is imperative for HSLP that the entire pre-analytical process be in the hands of experts, these being either laboratory technicians or professional phlebotomists (2,7,8).

The requisition is usually considered to be the responsibility of the attending physician. In general however, HSLP is served best when there is a close cooperation between the physician and the laboratory in deciding

the best choice of tests (2,9,10). Since requisition and interpretation of tests are closely related, these topics are discussed in the post-analytical Section on interpretation (S 4). Influences of the requisition pattern on laboratory costs will be dealt with in S 5.

The adequate preparation of the patient is of special significance in obtaining a good specimen (7,8). It has been repeatedly emphasized that standardization of blood sampling is a prerequisite for minimizing the variability of (haemocytometric) values. Differences in Hb, Hct, WBC and rbc values of 3 - 8% due to posture of patients during venipuncture have been described (5,11,12). These differences are recognized by ICSH (the International Committee for Standardization in Haematology) and IFCC (the International Federation of Clinical Chemistry). They are reflected in a joint document proposing an ICSH standard procedure for blood specimen collection for ref values either for people confined to bed or for those who are ambulant (13). Blood specimens obtained by venipuncture on ambulant persons while applying moderate and short term stasis (60 mm of mercury during 60 seconds) have 6 - 8% higher haemocytometric values than those obtained from the same persons in a recumbent position for 15 min. without stasis (11). Immediately after strenuous exercises increases of Hct, Hb and RBC up to 10 - 30% due to decreases of plasma volumes were found depending on the intensity and duration of the exercises and ambient temperature(5). Leucocytes even up to 50×10^9 wbc/l were found under these conditions (5)! Quite obviously recent blood transfusions will have their bearing on haemocytometric values. Notably higher MCV and RDW-values (in the Coulter S Plus II) due to bimodal rbc distribution are clearly demonstrable after transfusions into microcytic patients (14).

Large variations due to the time of sampling were observed for the wbc-count (5,15). Venipuncture specimens are strongly preferred to fingerstick blood. Fingerstick specimens not only have Hb-values discrepant to venous specimens, but, more importantly, the precision of blood drawing is clearly worse (about 3.5%) than with venipunctures (about 1.7%) (5,16-18). Comparable problems apply to WBC, RBC, Hct and PLT (5,16-19). Similarly, several studies have shown that Hb and Hct determinations in specimens from earlobe punctures give erroneous results and show unacceptable variability (5,20).

The collection and careful administration of a specimen for hcy deserves special attention (2,5,7). Possibilities of error are:

- incorrect patient and/or container identification
- usage of incorrect needles causing inappropriate flow rates (21)
- use of the wrong anticoagulant: e.g. oxalates and heparine are less suitable than EDTA
- delayed or inadequate mixing with the anticoagulant
- insufficient container filling leading to too high EDTA concentrations with concomitant MCV decreases (22)
- failure to examine for clots
- inhomogeneous-blood spill due to leakage of containers
- delayed delivery

Little attention has been given to detecting or minimizing laboratory blunders. Yet the clinician is much more likely to be aware of, and the patient more likely to suffer from, laboratory blunders than from small analytical variations. The "blunder rate" is variously estimated at about 2% (sic!) (23-25). No adequate solutions have yet been reported to further reduce this most serious phenomenon.

Long distance transport and storage of blood specimens are limited by the instability of whole blood (5,26-28). Cohle et al. (27) claim a stability of three days at 4°C provided the blood is intermittently mixed. However, these observations are challenged by Simmons, who mentions a stability of 8 hours at room temperature and of 24 hours at 4°C (28). Pneumatic tube transport does not influence the blood parameters (29).

A last, most important factor contributing to the overall quality of performance should be mentioned here. Technical expertise, experience, attitude, continuing education, organisation and general working conditions (job satisfaction, work-load, stress) of the personnel ultimately determine the quality of the laboratory (2,4,8,30,31). The impact of workload and stress is intuitively felt to be important. Performance in an EQA scheme has recently been inversely correlated with workload over a period of four years ($p < 0.01$) (32,33). The most frequent source of stress was found to be pressure for immediate results, the most intense stressor was shown to be lack of communication (34).

In conclusion, this Section on pre-analytical QA clearly demonstrates that the reliability of haemocytometric tests can be (grossly) affected by many factors beyond the actual functioning of the instrumentation. These factors should be fully appreciated and should be subject to QA no less stringent than that for the analyses proper (2,4,5,30,31).

3. Analytical phase

3.1 Basic problems of analytical QA

3.1.1. General problems of calibration and IQC

The classical study of Belk and Sunderman

in 1947 (35) on the accuracy of analyses in chemical laboratories led to a wide variety of activities in the area of laboratory performance evaluation. Many of the problems in achieving optimal precision and accuracy have been addressed in a series of recommendations on QC prepared by the IFCC (36-41). They deal with general principles and terminology (Part 1), assessment of analytical methods for routine use (Part 2), calibration and control materials (Part 3), internal QC (Part 4), external QC (Part 5) and quality requirements from the point of view of health care (Part 6). Moreover, several papers (42-45) and extensive overviews on analytical QA have recently been published (46-48).

The concepts of accuracy and precision are often elucidated using the results obtained by a champion-shot and an ordinary-shot, both using the same excellent and the same inferior rifle.(Fig. 1; Courtesy B.Leijnse - 44). It is a very good example. It illustrates how by craftmanship and superb quality of instruments the production process is improved by way of correcting the assignable cause of error, thus reducing the influence of chance on variation, and it also demonstrates that some stable system of chance causes is inherent in any particular scheme of production. However, an important difference between haem. and clinical chemical analyses on the one hand and markmanship on the other hand must not be overlooked. For the marksman the middle of the target is the true mark to hit. Therefore a rifle can be adjusted without any reasonable doubt, whereas in chemical analysis and hcy such a certainty cannot be achieved even with the use of ref material of the highest quality. One is not sure that the assigned value equals the true value (44,49).

The main statistical tool employed in IQC is the control chart, introduced in clinical

chemistry by Levey and Jennings in 1950 (50). This technique was derived from the pioneering work of Shewhart on IQC in industry in the thirties (51). The main principle of his method is stated briefly: Measured quality of manufactured product is always subject to a certain amount of variation as a result of chance. Some "stable system of chance causes" is inherent in any particular scheme of production and inspection. Variation within this stable pattern is inevitable. The reason for variation outside this stable pattern may be discovered and corrected. An excellent example of this train of thought is the well-known Youden technique applied in clinical chemistry and haem. to detect systematic errors (S 3.4) (44-45).

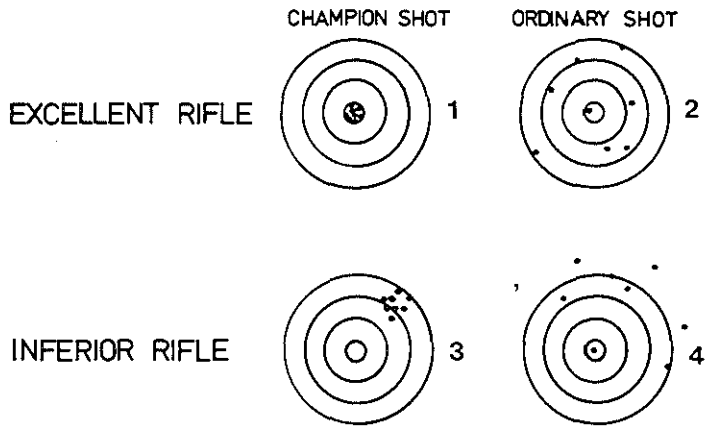

Fig 1. 1: accuracy and precision, 2: accuracy and imprecision, 3: inaccuracy and precision, 4: inaccuracy and imprecision.

In many respects, IQC programmes are similar for all disciplines, but in hcy there is a particular problem because of the difficulties in establishing ref preparations owing to the instability of blood cells, and,

except for Hb, the lack of suitable ref preparations. While precision has dramatically improved with the advent of electronic cell counters, the need for ref preparations for the blood count has become of paramount importance.

Unfortunately, the accuracy of blood cell counting cannot be simply tested by a pulse generator. While this testing can have value in verifying that the electronic calibration of specific instruments has not changed, it does not include testing of the aperture current in aperture-impedance systems such as Coulter counters nor does it detect the sensing zone changes which occur as a result of blockage, protein build-up, or variation in vacuum. Temperature variations will affect the blood flow rate through the sensing zones. The pulse shapes created by cells passing through these zones will be complex and varied. Constant square wave pulses as produced by pulse generators would give totally misleading information (52,53).

Most routine blood cell counting instruments are, in effect, comparators that can be adjusted arbitrarily (53,55) (Ch III, S 3.2). This means that to obtain a true measurement on an instrument which will be comparable with that on another instrument or by another method, it is essential to calibrate the instrument; this requires ref preparations with assigned values of defined accuracy (55). However, apart from the official ICSH Hb standard, ref preparations do not exist.

The urgent need for standardization and QA in haemoglobinometry was dramatically demonstrated in an international inter-laboratory trial undertaken by the Dutch Institute of Public Health in 1962. Participating laboratories, including the most eminent in Europe, reported the Hb conc of the same blood specimen between 11 and 18 g/dl (56).

The classical work of Van Kampen and Zijlstra in the early sixties on the standardization of haemoglobinometry brought a fundamental change in this field (57). Their precise and accurate optical density measurements and iron analyses on purified Hb solutions led to the establishment of the millimolar extinction coefficient of haemiglobincyanide (HiCN) at 540 nm as 11.0, while the relative molecular mass of Hb was calculated by Braunitzer to be 64458. On this basis, ICSH established recommendations for the Hb ref method and ref preparations (58).

In 1976 Van Assendelft et al. summarised the results of an inter-laboratory trial as follows: " In haemoglobinometry grave errors are still being made though an internationally accepted standardised method is available for the determination of the Hb content of blood " (59). They point out also that concentrated Hb solutions have become available recently, making it possible to control the dilution and conversion steps of the HiCN method. Using both HiCN ref solutions and concentrated Hb solutions, as well as checking the cyanide content of the reagent used, an acceptable intra-laboratory control programme may be set up. The within-run and between-laboratory imprecisions obtained were 0.6-0.9% and 2.9-3.3%, respectively (CV's). The work of Van Kampen, Zijlstra and others has brought substantial improvement in the quality of Hb measurements. Even the CV from 2.9 to 3.3 % found in the inter-laboratory trial mentioned is for a large part assignable. The photometry error has been estimated to account for about one-half of the total error, diluting errors for the other half. Elimination of these assignable causes of erratic fluctuation is possible and after that the process is under control. In that case a CV of 1% is within reach. The progress made in haemoglobinometry shows that the application of

ref methods and ref materials in inter- and intra-laboratory QA trials produces a substantial improvement. The assignable causes of quality variation can be separated out much better if ref values are used than if only the all-method mean value is available. This makes the diagnosis and correction of difficulties easier and improves the quality. Moreover the second object of Shewhart's technique is achieved. By identifying certain quality variations as inevitable chance variations, we know when to leave an analytical process alone and thus prevent unnecessarily frequent adjustments which tend to increase rather than decrease the variability of the process (44). The beneficial effect of standardization and IQC was also shown in the remarkable reduction in CV for the measurement of Hb in succesive years by laboratories in the United Kingdom: when the UK external quality assessment (EQA) scheme was started in 1963 the CV for this estimate was 7 to 8% but this has been progressively reduced to only 1.3% in the period 1975 - 1979 (55). The same dramatic improvement was observed in the United States College of American Pathologists (CAP) Survey Programme: whereas Belk and Sunderman observed in Pennsylvania in 1945 that less than half of the laboratories could come within 1 g/dl of the "true" Hb value (35), in 1975 the time had come to set the performance limits for Hb at approx. 0.3 g/dl (0.2 mmol/l) for good results, thus approaching the limits of known accuracy and precision of the ICSH-method (43).

The urgent need for calibration and IQC and EQA in cell counting and sizing was recognized during a workshop in cell counting instruments at the Physikalisch Technische Bundesanstalt (PTB) of the Federal Republic of Germany in 1981. Simultaneously, and under equal conditions, one fresh blood specimen was repeatedly (4x within-; 4x between-series)

analysed with different counters which were operated by manufacturers or service engineers. In contrast to the high precision of many instruments, the variation of the means is substantial (Fig. 2) (60). In a second series, control blood specimens from several manufacturers were analysed instead of fresh

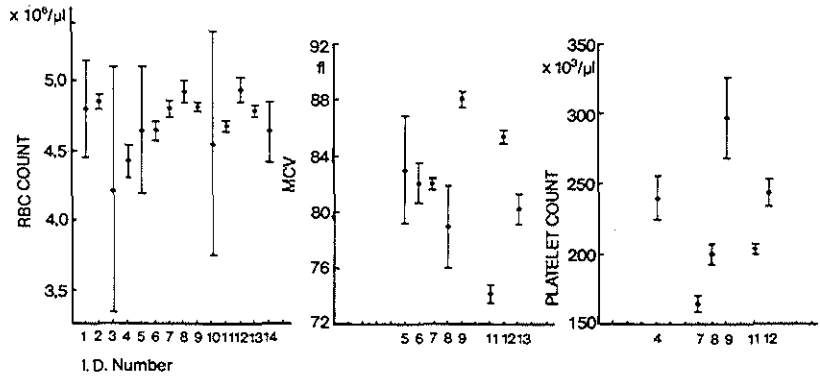

Fig 2. Instrument scatter: arithmetic mean and standard deviation for repeated measurements of rbc concentration, mean cell volume and platelet concentration. Experimental workshop in cell counting instruments (Berlin 1981) (Courtesy R. Thom, Calibration in haematology. In: S.B. Rosalki. New approaches to Laboratory Medicine. GIT Verlag, Darmstadt, 1981)

blood. The relative difference in % between the labelled value and the mean of measurements of all participants varied from -15 to 7.5% for wbc-substitutes and from -3 to 15% for MCV (60). The incompatibility between simulated wbc, stabilized rbc and reagents was apparently not recognized by several operators (cf Ch III, S 3.4.3.6).

There are serious difficulties in preparing ref preparations, and no true ref preparation for blood counts is yet available. Ideally the material should closely resemble

blood in its physical characteristics with regard to cell numbers, size, shape, electrical conductivity, homogeneity, viscosity and rheology (55). Unfortunately, materials which are sufficiently stable to serve as a long-term calibrator do not have the physical properties of blood, while natural blood is not sufficiently stable to be useful for this purpose (55). Moreover, an instrument calibrated for counting blood cells of one size, may not properly count cells of another size, if it is not linear in its response to cells of varying sizes (55). Furthermore, different instruments may differ in measurements of MCV on the same blood specimen, if they use different principles (Ch III) (55,66). This has been demonstrated in inter-laboratory comparisons of counters which use light scattering (Technicon Hemalog), conductivity (Coulter) and laser light diffraction (Ortho-counter) (55,66).

Materials which have been tried as potential standards fall into three main groups (55):

a. biological materials such as pollens, mould, spores, yeasts. They tend to be too heterogeneous with regard to size and have never proved suitable.

b. artificial materials such as polysterene latices and other plastic polymers. These are undergoing study by a number of (inter)national bodies (vide S 3.6).

c. natural blood cells which have either been preserved or modified by fixation in glutar-aldehyde or formaldehyde. Fixed cells have, for years, given consistently reproducible measurements of cell numbers and size distributions. Unfortunately, there is a major disadvantage in using these cells as a ref preparation. There is a tendency for fixed cells to clump and to adhere to the walls of the containers, so that unless the sample is

meticulously resuspended, non-homogeneity may arise and lead to loss of accuracy and precision. In fact, undiluted fixed cells are not suitable for use in fully automated systems (61). Furthermore, the rigidity of fixed cells results in erroneously high micro-haematocrit values due to increased trapped plasma.(cf Ch III).

Preserved natural blood cells are not stable enough to be regarded as a long-term calibrator. However, these materials are useful as controls to check the precision and reliable functioning of a cell counting system over relatively short periods of time (55,61-63).

Ch IV extensively deals with attempts to prepare control materials for IQC in hcy. They serve three basic purposes of IQC.

a. Process control, to be executed "ad hoc" by the technician by regularly running the material and instantly judging the results obtained (2,4).
b. Retrospective IQC by the haematologist or by the "Q.C. officer" by evaluation of the proficiency attained both in within-day and between-day periods (Shewhart charting) (2,4,8,30,46,48,50).
c. Blind controls to assure optimal IQC. This is done by regularly interspersing and evaluating results of other preparations of known composition, disguised as routine patient specimens within routine sample-series. The tendency to (solely) delegate this type of control to EQA schemes threatens the alertness and readiness of action of the laboratory (39,100).

In short these materials are intended to contribute to the essence of IQC: continuous self-auditing and continuous attempts at improving performance.

3.1.2 Specific problems of IQC of individual blood cell parameters

3.1.2.1. Errors due to abnormalities of the blood specimen

Cornbleet (22) has recently reviewed the sources of error in cell counting and sizing due to abnormalities of blood specimens. Serious rbc-count and rbc-parameter errors may be caused by specimens with a. cold agglutinins (4,22,64) b. positive Coombs' test (22,65) c. paraproteinaemia, opaque or strongly coloured plasma, d. haemolysis e. high WBC, f. giant platelets, g. microcytic rbc, h. clotting etc (22). The main sources of erratic results in wbc counting are a. cold agglutinins (22,64) b. aggregates (22,69,83,84) and c. the presence of nucleated red cells (22,83,84). Errors due to carry-over have been resolved in modern flow hcy instruments (66). For the cause of brevity, we refer to References (22, 64, 83, 84) for a full account on the subject. Spurious elevations of electronicly determined MCV and Hct caused by hyperosmolarity have recently been reported in hyperglycaemia and diabetic ketoacidosis (70,71). An under-estimation of 6-7% of Hct in polycythaemic patients was described for Coulter S and S-Plus instruments compared with micro-haematocrits corrected for trapped plasma and a radioisotope dilution Hct (72,73). This is in accordance with the observations of England and Down (74) and Arnfred et al. (75) that the MCV is not accurately measured by electronic counters when the rbc are hypochromic and microcytic (see Ch III). Spuriously elevated plt counts (mostly accompanied with abnormal MCV and/or instrument error code flagging) were described in cases of acute burns due to microspherocytosis (22), in haemolytic hereditary elliptocytosis (76-78) and in leukaemia due to leukaemic blast or

megakaryoblast fragmentation (22,78,79). The most frequent cause of spuriously decreased plt- counts is plt aggregation either due to difficult blood sampling or occurring spontaneously or due to plt to wbc adherence (platelet satellitism) (22,69,79-83).

A particularly important source of error in plt (and wbc) counting is pseudothrombocytopenia due to plt aggregation, although its incidence (predominantly in EDTA) is only approximately 1%. A falsely low plt count may lead to unnecessary treatment or to the unjustified withdrawal of needed drugs. Antibodies of all major immunoglobulin classes and all IgG subclasses can be found in EDTA-dependent pseudothrombocytopenia (67). They are probably not directed against EDTA itself, but may be directed against a hidden antigenic determinant of plt membrane glycoproteins (GP) that are exposed in the presence of EDTA (67). This is strongly suggested by the fact that plt from patients with Glanzmann's disease (which are deficient in the GP-complex designated GP II b or III a, or both) do not react with the (aggregating) antibodies (67). It is shown that EDTA dissociates the GP-complex. Thus EDTA may expose a neoantigen that is then the basis of the "pseudo" effect (67).

Fortunately, plt aggregates can readily be detected in instruments such as the Coulter Models S-Plus II-V as a shoulder in the extreme left portion of the wbc distribution histogram (83). Current anticoagulants (EDTA, citrate, heparin) all result in plt aggregation, though at different times after sampling (68,82). Aggregation can be prevented (for at least 3 hours) by drawing (4.5 ml of) blood into (0.5 ml of) an acid citrate buffer of pH 4.65, resulting in a final blood pH of 5.50-5.60 (82).

3.1.2.2. Errors due to method or instrument deficiences or malfunctions.

An extensive compilation of errors due to method or instrument deficiencies or malfunctions for Coulter S-Plus instruments has been issued (84).

3.2 Approaches to IQC.

Because of the unsuitability of human blood as a ref and control material in hcy, many IQC methods have been proposed in the past. Methods primarily aimed at monitoring the precision of the analytical process are summarized in Table I (57,85, modified and updated).

Bull (87) was one of the first to show how the periodical determination of the mean MCV, MCH and MCHC could serve as a major contribution to IQC of automated analyzers, due to the approximate constancy of these patient parameters.

There is general consensus now that no one IQC tool can provide all the information necessary for an effective IQC system. Consequently a combination of IQC materials and computation of patient values are to be preferred.

Computation of patients' data obviously can be best accomplished by computer. However, even in case where a computer is not available occasional off-line implementation of this valuable technique should be advocated, although it is time-consuming and has several inherent deficiencies (94). A large number of commercial IQC materials are in the market (97). Home-made IQC materials are less expensive and are to be preferred (Ch IV). However, constant vigilance and judicious observation as to the quality of the IQC

Table I Historical survey of approaches to precision control in haemocytometry

	Control method	Advantages	Disadvantages
I	commercial control materials (Ferro 1969) (86)	well-known concept, easily implemented	dependence on external source of material; large costs
II	patient rbc-indices (Bull et al. 1974) (87)	rbc-indices are an inherent "standard"; low cost	computer facilities necessary for optimal application; insufficient by itself;no check on WBC and PLT
III	truncated adult mean parameters; combination with accurate Hb-determination (Rutten et al. 1975) (88)	as II	computer facilities necessary; less suitable for WBC and PLT
IV	random patient duplicates (Carstairs et al. 1977) (89)	simplicity, especially for random error detection; precision well documented; low cost.	insufficient by itself
V	moving patient modes (Pragnell-Johnson 1977) (90)	less affected by abnormal results than II	as II, more complicated calculations
VI	(commercial) control plus patient rbc indices (Lappin et al. 1979) (91)	this and similar techniques give optimal information	computer facilities necessary for optimal application
VII	own preparation (Cavill et al. 1979)(92)	inexpensive	difficult to prepare
VIII	commercial control + modified average of normals technique (Talamo et al. 1981) (93)	more sensitive than average of normals method; as VI	as VI
IX	control materials, calibrator, and patient values (Bull et al. 1982)(94,95)	as VI	as VI

materials is needed. This is best accomplished by having another IQC material constantly available. Provided HSLP requirements are met (see S 3.3) precision in hcy can be kept within narrow limits.

A major problem, however, is the determination of assay values of blood preparations that are used as standards or calibrators. This will determine the accuracy of the measurements.

The calibration of fully automated multichannel blood cell counting instruments has been approached in essentially three ways (98).

a. Some have advised the use of fresh whole blood, analyzed by manual or semiautomated techniques, as the most valid method. Coulter advocated numerous repetitive measurements of fresh whole blood by routine manual procedures. The multiple assays (usually 20) are designed to absorb the inherent imprecision of these methods and to yield an acceptable average or mean value for use as a calibration point. Proponents of this technique, however, caution users to investigate manual procedures further if unlikely or unmatched results occur. The advantage of this approach is the ready availability of such methods because most technologist-operators are familiar with them. The disadvantages are the large quantity of blood required and time of the technologist, as well as the very real possibility that the mean value obtained may not be accurate (specific sources of potential error for individual blood parameters are discussed in S 3.1.2).

b. An alternative approach to fresh whole blood calibration was described in 1977 by Gilmer et al. (99), whose practice is to perform a few repetitions (three to five) of carefully performed calibration techniques. They defined calibration methods as procedures performed with greater care and precision than routine manual techniques, yet differing from primary

ref methods, which generally require research facilities and instrumentation for proper execution. These procedures do require that quantities be directly measurable by materials, glassware, and instrumentation of known or verified accuracy. Although this approach has proved to be sound theoretically, it has been considered to be too tedious and time-consuming for practical use in the routine haem. laboratory.

c. The third approach is probably the least well documented and yet by far the most widely used. It employs preserved whole blood cells prepared for QC and involves accepting the manufacturer's assay values as valid calibration points. This method is probably agreed generally to be the best approach for initial calibration of a new or recently serviced instrument. If a second, different control material is then run as a routine sample and results closely match its assay values, the instrument may be assumed to be reasonably well calibrated. This should be checked in one or more EQA schemes (see Section 3.4). The advantage of this pragmatic approach is, of course, ready availibility and maximum savings of time and trouble. The disadvantages are the dependency on the manufacturer's assay values at the time of production and, even more important, the lack of assurance that these materials wil survive a wide variety of shipping hazards. An extension of this is that the user must also stringently follow manufacturer's directions for storage and handling. It also is a source of concern that a variety of preservatives and substitutes must be used to simulate samples of fresh whole blood, and this is compounded when routine manual techniques are applied to verify values of preserved control material (Ch III, S 2.3.4).

The problems described here could be

alleviated if substantial progress is made in value assignments, as undertaken by (inter)-national agencies. These are considered in S 3.6 and S 3.7.

HSLP-techniques (cf S 3.3) and participation in EQA schemes will ultimately guarantee optimal laboratory results, in conformity with the present state of the art in QA.

3.3. High-Standard Laboratory Performance in the analytical phase.

HSLP in the analytical phase can be achieved if a single instrument has been evaluated according to the (tentative) ICSH protocol (140) and if the following operational requirements are met in the daily routine of a particular laboratory (2,4,8,14,46,50)

- the presence of standard operational procedures, filed in an Instruction Manual.
- thorough preventive maintenance and electronic checks of the instrument. Administration in a maintenance log book.
- frequent instrument calibration and linearity checks.
- continuous IQC with control materials preferably in conjunction with the use of a computerized patients' data programme (S 3.2). (process control).
- technician's inspection of individual specimens and their measured results. (process control).
- logging and graphing or plotting of the results to detect shifts or trends (preferably Shewhart-Levey-Jennings charting: retrospective IQC).
- regular interspersing of blind controls and evaluation of the results (retrospective IQC) (39,100).

- regular participation in EQA schemes (S 3.4).

3.4 External Quality Assessment (EQA)

External quality assessment (EQA) is the objective evaluation by an external agency, under direction of a professional peer-group of the performance by a number of laboratories with respect to material supplied for specified tests. Interlaboratory trials play an important role in maintaining laboratory proficiency. They form the basis for EQA schemes by means of which the technical skill of individual laboratories and the efficacy of their equipment can be assessed (61). In addition, they provide data for assessing methods for identifying the limitations and faults of a particular method, type of equipment or reagent (61). The most important function of an EQA scheme is to provide a means for ensuring comparability of results between laboratories. (61) This is especially important for analytes for which there is no "absolute" reference material available. In this case, without an EQA scheme, the individual laboratory cannot judge the comparative accuracy of its performance, even if it has a well established IQC system. An EQA scheme provides specimens for inter-laboratory trials and values can be assigned to these by statistical analysis of the results of all participants. This provides comparability and standardization by consensus, even if it is not possible to establish and verify the results as being absolutely true (S 3.1.).(61) At this time several regional, national and international programmes are organized. In this Section we shall demonstrate some of the recent results of a regional scheme. Fig. 3 depicts the Youden plots of the 5 basic parameters in the regional Leiden programme of October 1984. Fig. 3 shows that the spread in Hb, MCV and RBC is mainly due to

systematic errors, whereas the high CV's in WBC (9-10%) and particularly PLT (12-14%) are due to both systematic and random errors. The occurrence of relatively large systematic errors in the Hb determinations of the Leiden trial shows that even the existence of a ref method and a haemiglobincyanide ref solution does not guarantee optimal laboratory comparability. This was earlier described by Van Assendelft et al. (59) (S 3.1.) Even the simultaneous availability of stable Hb-solutions for IQC (59) apparently has not led to optimal Hb results. Undoubtedly this can only be due to insufficient application of the basic elements of IQC, as described in S 3.1.

Table II shows the order of magnitude of CV's of the different parameters in various EQA schemes. It reflects the state of the art of EQA in hcy. In the clinical situation regional comparability seems more important than (inter)national comparability. Consequently, higher standards should be applied to the results of regional EQA schemes. In my opinion the results obtained in the two regions mentioned are unsatisfactory for WBC and PLT and rather poor for the other parameters. I feel that one or more of the following pre-requisites should be met in order to improve the results in EQA schemes.

a. The routine use of counting chambers should be abolished because of their poor reproducibility and liability to negative bias.
b. Ref materials should become available.
c. IQC should be intensified along the lines described in S 3.1 (process control, retrospective IQC, blind controls, using materials such as published in Ch IV).

It should be re-emphasized, however, that the outcome of these programmes is not completely similar to that of clinical specimens encountered in practice. Obviously, an EQA scheme by itself is not a complete

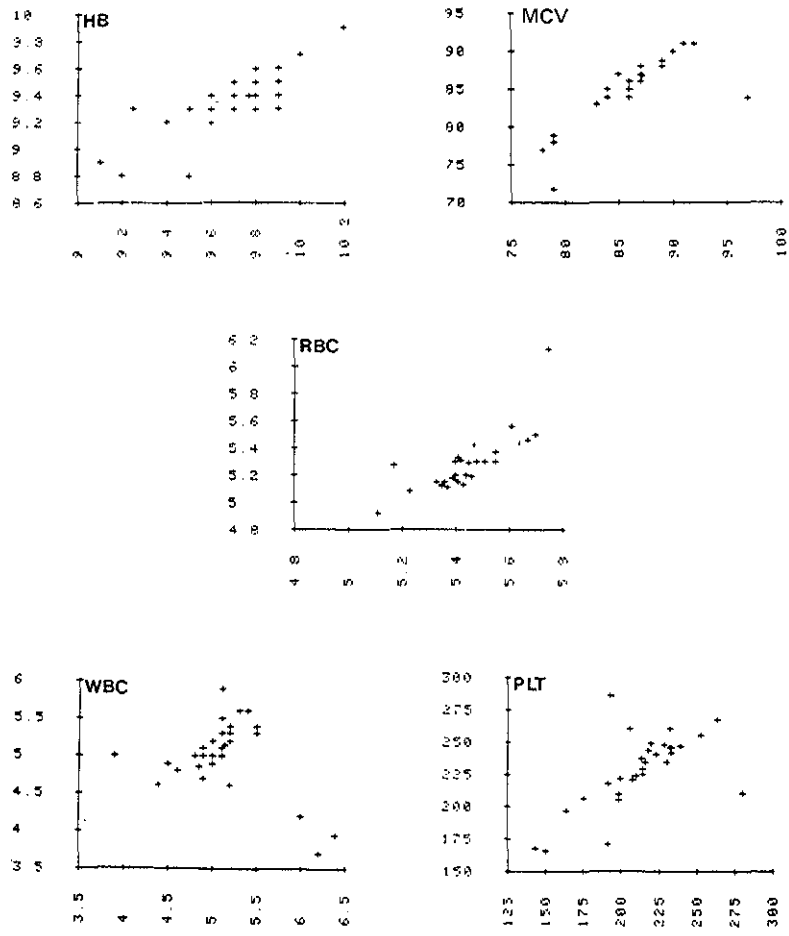

Fig. 3. Youden plots of the 5 basic parameters in the regional Leiden EQA scheme (October 1984) (Courtesy J.F.v.d.Burgh, Central Clinical Haematology Laboratory, Academic Hospital, Leiden, The Netherlands)

system of QA. The entire spectrum of pre-analytical variability (S 2) escapes control by these schemes. Much of the variability in result reporting is not measured by them. Other objections to EQA surveys, especially applying to hcy are : (102)

a. the distributed material may behave differently from the biological specimens normally investigated by the laboratory, and hence give a misleading impression of the laboratory's normal level of performance;

b. the distributed material may be handled by the participating laboratories in a way different from its routine practice, so that the impression gained in the survey bears little relation to the day-to-day level of the laboratory's performance. (ideally, EQA should be "blind", with the distribution specimen treated in exactly the same way as a routine specimen, but this is not always easy to arrange and/or may be impossibe);

c. the distributed material may deteriorate in transit to a different extent for different laboratories;

d. laboratories may consult one another about their results on the distributed specimen before submitting these to the organizer, thereby conveying a falsely favourable impression of between-laboratory consistency;

e. there is considerable doubt as to whether either the consensus mean or the results obtained by ref laboratories constitute a scientifically impeccable base point for comparison with individual participants' results; this applies especially where a predominance of use of a particular apparatus in the region gives a "machine-bias" to the results (66,98; Ch III) and

f. surveys can cover only a limited number of tests, which may be such a small fraction of a laboratory's range as to be unrepresentative of its total work.

Ways of overcoming or minimizing these and other difficulties in the survey method are suggested in a series of WHO recommendations (102).

Table II Typical average coefficients of variation of five basic parameters in 2 regional (Dutch) and 3 national EQA schemes (Compilation of method groups).

EQA Scheme	n	nature of control material	date/period of analysis	WBC	RBC	Hb	MCV	PLT
Leiden	28-42	one human EDTA-blood	mean of 12 trials (1983)	8-10	4	2-3	4	13-15
Venlo (Ref. 101)	15	mean of 10 human EDTA-bloods	1980 - 1981	6-9	3-4	2	-	10-12
Dutch	122	(mean of) 4 human bloods	August 1984	8-11	4	2	-	11-13
German "Ringversuch"	392	human rbc;CPDA-plasma; fixed goose rbc; EDTA-stabilized plt	October 1984	6-12	3-8	2-4	3-7	10-35
French "Etalonorme"	3400	- -	November 1983	6-9	4-5	3	4-6	-

Although EQA is not meant to abandon rigorous IQC in the mistaken belief that EQA provides an adequate check on daily work, the time that elapses between analysis and receipt of the results should be as short as practicable. Rapid feedback enables the laboratory to investigate recent problems, if any, however difficult and sometimes impossible this turns out to be in practice. Too long a delay will not only dissipate interest but will

also diminish the possibility of identifying causes of inadequate performance. The main value of EQA should be seen as educational, its aims should be to help participants to maintain and/or improve performance in their laboratories. Energy should be directed both within the laboratory and within regional discussion groups to endeavour to identify accurately means for improvement in performance and factors leading to divergence from the national norm or the ref results. The main purpose of these regional groups is therefore mutual education and training (24,102).

3.5 Analytical goals.

Although from the point of view of the analytical chemist precision and accuracy must be of the highest attainable standards, in daily practice, there is considerable controversy on the formulation of analytical goals. Some (103) assert that the precision performance of nearly all analyses, including hcy, exceeds the medical requirements, as originally published by Barnett (1). Others, including IFCC (41), state that there should be varying levels of expectancy for analytical goals for different clinical uses, although practice indicated that such is not the case (41,104). Several studies (104-106) have alluded strongly to the very subjective quality of clinical opinion. One of them states that it is generally accepted that current goals are derived best from biological variation (105) (see S 4.2). The clinical view of analytical goals is, therefore, not easy to assess. Moreover, once defined, medically pertinent analytical goals must not be static or fixed. Goals must be modified according to clinical, scientific or analytical progress (104). Others state that it is scientifically unsound to monitor an objective process, such as an

analytical procedure, with subjective criteria, such as medical requirements (107).

The fundamental question here revolves around the role of the clinical laboratory in patient care. Is it to provide quantitative data on patient specimens that is obtained by the most reliable "state of the art" analytical techniques, or is it to provide more crude data merely to confirm clinical impressions ? In my opinion, the clinical laboratory should serve as a scientific adjunct to patient care, available to physicians to screen for, diagnose, and/or monitor, disease processes in their patients. In this capacity, the clinical laboratory must provide the most reliable quantitative data possible (as far as costs permit), monitored through objective QA procedures.

Cell counts approximate to the Poisson distribution, so that it is possible to calculate the "ideal" CV of the number of cells from the square root of these counts (108). Haynes shows that in his experiments the observed CV approximates to this theoretical CV in plt counting, indicating that the precision is limited primarily by sample size and counting time (109). Coulter specifies the precision for the Model S-Plus II as CV's of <= 2% for WBC, <= 1% for RBC and <= 3.3 - 6.6% for PLT (all dependent on the particle conc). They are based on 31 determinations of the same sample and verified by numerous data sets in various locations (14). The established precision specifications for a normal patient sample in Ortho's ELT-8 are slightly greater: <= 3% for WBC; <= 2% for RBC; <= 3% for PLT (Ortho Manual).

3.6. Value assignments by (inter)national bodies

In previous Sections the need for

standardization in hcy is extensively described. Establishment of ref materials and methods requires a collaborative study and consultation with experts from many countries.

By universal agreement of national governments, the World Health Organization (WHO), acting through its Expert Committee on Biological Standardization is responsible for "establishing international biological standards, reference preparations and reference reagents for these biological materials used in prophylaxis, therapy or diagnosis of human (and some animal) diseases that cannot be characterized adequately by chemical and/or physical means alone" (55,110). Over the past decades an increasing number of other organizations have also become active in biomedical standardization. A recent meeting under the aegis of WHO identified at least 28 such organizations (55). In haematology standardization has largely been undertaken by ICSH. Since its founding in 1964, ICSH published several "Recommendations" (111-112). The successful standardization in haemoglobinometry is a good example of ICSH-WHO interrelationship and cooperation (55).

Recommendations for ref methods and ref preparations for blood cell counting and sizing are the subject of study by an expert panel of ICSH. The problems that they encounter are not easy to solve. It is (up to now) not possible to develop ref preparations that behave in the same manner as fresh blood when analyzed by cell counters (111). Moreover, particle counting and sizing in general is a basic technological problem (60,109,139). Accurate counting can, among other causes, be seriously hampered by particle aggregation and/or adsorption or by count loss due to counting coincidence. Furthermore, Poisson statistics demand that at least 10.000 particles be counted, if precision is to be kept below 1%.

These and similar problems as well as those of the actual counting devices are discussed in Ch III. In haematology, the accepted ref count is produced by digital flow hcs e.g. a Coulter Counter ZBI, with manual large scale volumetric dilutions counted above or between specified size thresholds (14,60,97,114,139,140). The Coulter thrombocounter is now recognized as (one of the) plt ref counter(s) (139). In this type of digital instrumentation the cell conc is determined directly by counting the number of particles in a given volume. This volume is a calibrated liquid column between a start and a stop sensor (60).

Of the many particles mentioned in S 3.1.1., latex particles are the most promising potential ref preparations. Spherical polystyrene latex particles in a series of defined sizes, with a claimed CV of 2 to 3% have recently become available. These particles appear to be well-dispersed with little aggregation and preliminary studies have confirmed that at least some batches are relatively monosized (60,113).

Particle sizing can be performed with high accuracy by means of light microscopy provided the monosized latex beads are aligned in a long array (60)(Fig. 4), to prevent resolution problems (109). Electron microscopy values are said to be unreliable (60,109) due to size changes in sample preparation (109). Nevertheless, Coulter's ref material is a latex sized by independent workers using electron microscopy, but not certified by a competent independent authority (139). The values have been verified, inter alia, by techniques traceable to the American National Bureau of Standards material 1003, but there is still a great need for a certifying authority, e.g. ICSH, ECCLS, BCR or WHO, to produce a suitable ref material to which the commercial secondary ref materials can be related (139).

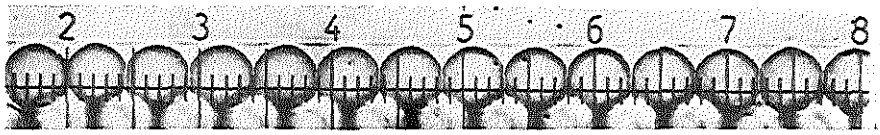

Fig. 4. Monosized Latex beads (diameter = 7.0 um aligned) in special glass capillary tubes. Partial image of the array. The mean particle diameter can be determined with high accuracy by measuring the longitudinal displacement of the microscope mechanical stage under control of cross hair eye-piece and photoelectronic sensor (accuracy in the total range 0-30 mm +/- 0.001 mm. (Courtesy R.Thom, Calibration in haematology. In: S.B.Rosalki. New approaches to Laboratory Medicine. GIT Verlag, Darmstadt, 1981)

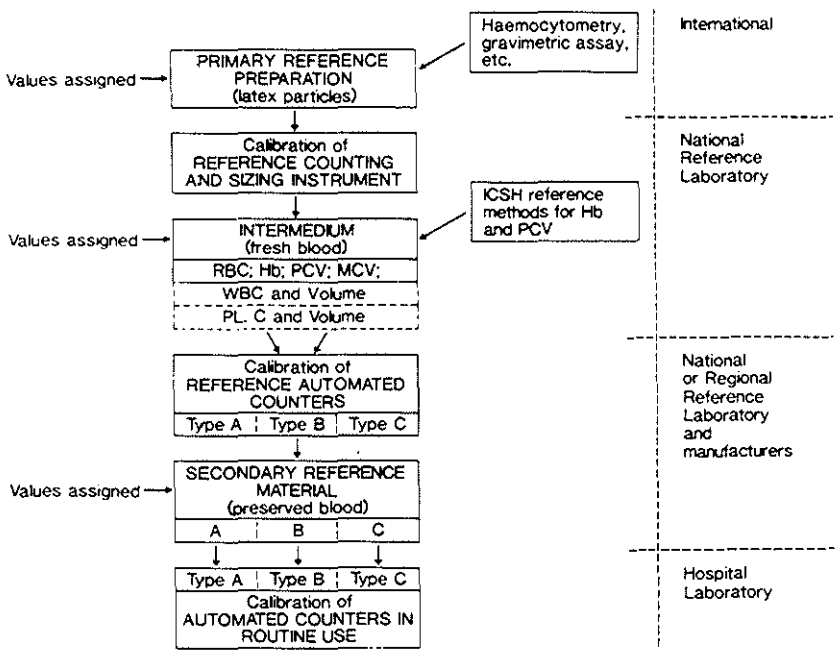

Fig. 5. Flow sheet of scheme for assigning values to reference materials (Reprinted with permission from SM Lewis. The philosophy of value assignment. In: DW van Assendelft, JM England. Advances in hematological methods: the blood count. Copyright CRC Press, Inc, Boca Raton, Fl, USA; 1982)

Latex beads can be expected to become a certified ref preparation applicable to cell counting and sizing with the Coulter principle, the latter in consideration of the shape-factor of rbc. (spheres = 1.5; rbc = approximately 1.1; see Ch III, S 3.4.3.2 (60)).

A detailed scheme for assigning values to secondary ref materials has recently been described (113). Fig. 5 shows the flow sheet of such a scheme, based on a study sponsored by BCR, Brussels (113). To translate the primary ref preparation into a secondary ref material for measuring blood specimens, it is proposed that fresh blood is used as an intermedium and preserved blood as the secondary ref (113)(Fig. 5).

A most important, in principle readily applicable scheme for assigning values to secondary ref materials has recently been published by the British Committee for Standardization in Haematology (BCSH) (114). The protocol is based on using an automated blood counter to compare WBC, RBC, Hb, Hct and rbc- indices of an IQC material with those of (three) fresh bloods, whose values have also been measured directly in a Coulter Counter Model ZBI and by ICSH ref methods for Hb and Hct (114) (Table III). With the knowledge of :

a. the directly measured values of the fresh bloods and
b. the ratios on the automated counter (fresh blood value/IQC material value), it is then possible to precisely assign values to the IQC material for that particular automated counter. The Committee suggests that at least three institutions (hospitals) participate in order to average out inevitable inter-institution biases (114).

In both schemes the standardization procedure will be repeated at regular intervals, the frequency being determined by

the stability of the secondary ref materials. A similar procedure can be adopted to assign values to preparations intended as secondary ref materials for plt.

3.7 Proposals for calibration in daily practice

In my opinion, the current practice of (initial) calibration with IQC materials (S 3.2, p.36 approach c) should be maintained, for pragmatic reasons, despite the theoretical objection to using IQC material for calibration purposes. As laboratory practice has

Table III. BCSH scheme for assigning values to IQC materials

Participants : 3 institutions (same automated counter)	
3 fresh bloods	- direct values by Coulter ZBI-counts, by ICSH-Hb method and by ICSH-Hct method.
Same 3 fresh bloods) and IQC material)	- 3 ratios blood/IQC material on particular automated counter
Assigned value	= geometric mean of direct values / geometric mean of ratios

persistently shown, individual laboratories hardly have a realistic alternative (S 3.2,p.35 approaches a and b). Counting chambers have no real place in accurately counting blood cells (Cf Ch III, S 2.1). As already mentioned in S 3.6, in practice digital flow hcs have been recognized as the instruments of choice for counting blood cells (cf Ch III, S 3.2).

However, they are generally not available in hospital laboratories, since most routine flow hcs are analogue instruments (comparators). The ICSH-Hct method is too cumbersome for the individual laboratory (cf Ch III, S 2.3). The above mentioned objections would be obviated by the application of the BCSH protocol briefly described in S 3.6 and Table III. In my opinion, it is the only currently available method potentially able to improve interlaboratory comparability by further approximating "absolute" values. However, this method requires intensive regional or national cooperation.

4. Post-analytical phase

4.1. Recording and reporting.

Transcription errors are avoided by printing the results directly on the request forms, as used with multiparameter instruments (66) or by on-line computer reporting. Normal blood count values are usually reported without any type of comment. Stat values are usually reported by telephone, by preference directly to the requesting physician. Nothing is gained by perfect laboratory performance, if the clinician does not make good use of the results. Considerable efforts must be made to communicate with the physicians on all aspects of laboratory performance, especially on the interpretation and significance of hcy results. This is dealt with in the next Sections. Ideally, clinicians should list recently administered drugs on the request forms and/or bring up for discussion with the laboratory head any unexpected, contradictory, or otherwhise problematic results as well as unusual or unacceptable delays in reporting. An agreed procedure should exist for this purpose and should not be allowed to lapse or to be

replaced by the doubtful expedient of requesting repeat analyses without prior consultation (41). A Hospital Information System could play an important role in the promotion of communication (115).

> "It is not enough to have great qualities; one must make good use of them." La Rochefoucauld (1613 - 1680) Maximes no. 159 (97)

4.2. Aspects of the interpretation and significance of haemocytometric results

4.2.1. Intra-individual variability: significance of successive counts (108; 116-118)

Once methodological constancy has been ensured and quantitative knowledge has been gained of the precision of measurements in the modern very precise flow hcs, an attempt can be made to gain insight into the intra-individual variability of the various parameters in healthy individuals. This was first reported by Statland et al. (116) and more recently by Cavill et al. (117) and by Costongs (118). This enables the laboratory to give the clinician an indication when a difference between two sequential results is pathologically significant. This is unrelated to the better-known concept of ref values (S 4.2.2) but concentrates on detecting haem. changes in a single individual.

Despite small but significant differences due to sample techniques (S 2) evidence indicates that the Hb-conc in a single individual is remarkably constant, the maximum day-to-day CV being less than 3%. At a Hb-conc of 9 mmol/l, this means that two successive measurements should be within 0.25 mmol/l of the mean and that the difference between such

measurements should not exceed 0.6 mmol/l. Variations less than that should not necessarily be attributed to pathological change. However, it is still possible for smaller changes to be pathologically significant, if a succession of specimens show a consistent trend (108). In contrast, Costongs (118) presents evidence that a month-to-month change in Hb-conc of 1.4 mmol/l, at the 8.5 mmol/l level, has a probability of being pathological of (only) 90%: the so-called critical difference dk90 = 1.4 mmol/l. I feel that this conspicuous finding requires confirmation before it can be generally accepted.

The day-to-day CV for RBC is reported to be equally less than 3%. Thus at a red cell count of 5.00 x 10^{12}/l no two counts should differ by more than 0.30 x 10^{12}/l unless the balance of the rbc production and destruction is disturbed. (108,117)

On the other hand, the CV for wbc-counts may be of the order of 15%. This means that for wbc-counts in the region of 7 x 10^{9}/l the count for any individual may very well vary by 2.2 x 10^{9}/l between successive counts as a result of chance alone. Although exercise has been said to increase the wbc-count (5), a systematic study provided no evidence of any significant relationship between activity prior to blood sampling and the measured wbc-count (108,117).

The stability of the rbc indices is well-known and is used in analytical QA, as we have seen (S 3.2). The inter-individual CV's for MCV, MCH and MCHC are estimated to be approximately 4% (94). In a patient population of a typical, acute care, general hospital the variation is only slightly greater, in the order of 6% (94).

In normal subjects there may be a CV of 13% on plt-counts, although the hour-to-hour variation is generally less than 2%. Thus in

the region of a plt-count of 120 x 10^9/l results on successive days might be expected to differ by up to 30 x 10^9/l. When the count is made by a well controlled, automated method it would be unwise to attribute any difference of less than 30 x 10^9/l between successive counts to pathological change. For manually counted plt it might be prudent to double that figure, in case the counts are not scrupulously performed (108). Costongs found a significant upward trend during one day for WBC, PLT and MPV (118). Furthermore, he observed a critical difference in dk90 during a six-month period of 0.83 x 10^{12}/l for RBC, 3.6 x 10^9/l for WBC and 93 x 10^9/l for PLT, all these values again being higher than those mentioned above.

4.2.2. Inter-individual variability : reference values.

The human organism is subject to variations caused by physiological processes, genetic differences, diseases and environmental factors. A rational interpretation of haem. results demands knowledge of these variations in the individual under study (S 4.2.1.) or in an adequately defined set of ref individuals. It will be evident that intra-individual ref values are more suitable than inter-individual ref values (118). However, the former generally are not (yet) available in daily practice. Alternatively, an important task for the laboratory is to provide reliable sets of inter-individual reference values. Customarily, laboratory test results are interpreted by comparison with traditional but inadequately defined "normal" values. This ref information too frequently is the weakest link between the laboratory and the clinician. W.Elion-Gerritzen extensively described the remarkable dispersion of "normal" range values, action levels and medically significant changes among physicians

(106). However, the proper interpretation of laboratory data depends, inter alia, on the quality of ref information available to the physician. Increasing awareness of the biological changes in physiological and pathological processes demands a more precise and comprehensive interpretation. A rational approach to providing a sound basis for interpretation of observed values calls for a theory which describes the principles and procedures for selection of ref populations and definitions of ref values. There is need to evaluate alternative approaches to the generation and application of ref values appropriate for various purposes (119).

ICSH and IFCC are preparing a series of joint recommendations on the theory of ref values (113,119,120). Nomenclature for describing the relation of observed values to ref values is unambiguously formulated and proposed for universal acceptance (120).

Establishment of "absolute" ref values is impeded by the lack of ref methods and standards. This is one of the main reasons for the great variety of published ref values. A practical approach to the calculation of ref values is the use of the hcy results of an unselected (with respect to health characteristics) patient population, as routinely produced in a haem. laboratory. Using the bulk of hcy data generated every day in laboratory routine practice is attractive for the following reasons:

a. it obviates the great difficulty of selecting a ref population which is homogeneous with respect to certain health characteristics;
b. analysing a great number of samples taken from members of a ref population is costly.

Naus (121) describes and compares five different calculation methods using unselected patient data. From this comparison the method described by Bhattacharya appears to give the

best results and it is relatively simple. Naus et al. (121,122) extensively describe the concepts and techniques of the Bhattacharya plot. In another study (123) significant sex differences were found for all parameters while age differences were present in ref values of WBC, RBC, MCV and MCH.

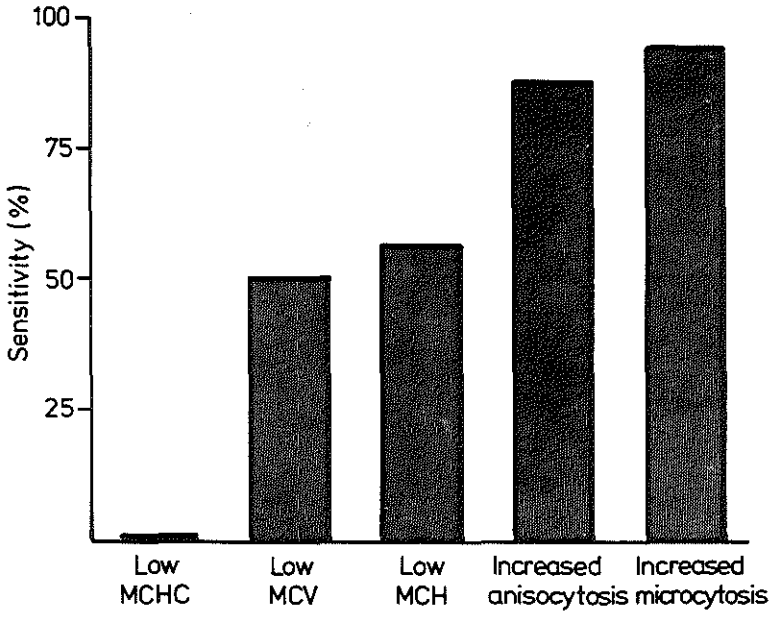

Fig. 6. Sensitivity of various haematological parameters for detecting minor degrees of iron deficiency (Reprinted with permission from JM England et al. and Blackwell Scientific Publications, Oxford; Br J Haematol 1976; Ref 125)

4.2.3. Clinical efficacy of haemocytometric tests.

Two basic concepts are involved in the validation and implementation of a clinical test (124). The first step is to choose the best test from among the available

alternatives. This means establishing the relative ability of the tests to make correct clinical classifications, as reflected in their sensitivity and specificity. A simple example is given by the sensitivity of various haem. parameters for detecting minor degrees of iron deficiency (125) (Fig. 6). Note that the MCHC has lost its diagnostic sensitivity with the advent of flow hcs (126). With manual counting methods and a Hct derived by centrifugation (in Wintrobe tubes), the MCHC is low in iron deficiency and other conditions associated with small red cells and concomitant anisocytosis. This is caused by falsely high Hct's due to increased trapped plasma in anisocytosis; hence an apparent fall in MCHC is obtained from the ratio of Hb and Hct (127-129). The discriminatory function has been taken over by RDW (83).(cf Ch III, S 3.4.3.4)

When the best test is selected, the second step is to choose the appropriate decision level for a particular clinical situation. This requires consideration of both prevalence of the disease (Table IV readily shows that a particular test has a higher predictive value when the disease occurs with a higher prevalence) and the medical (and economical) consequences of false-positive and false-negative results. This concept, based on Bayes' theorem is exemplified by Fig. 7, that illustrates how sensitivity and specificity are altered by the selection of an upper limit of normal. Fig. 7 shows the classic overlapping distributions of WBC in patients classified into septic and nonseptic based on blood culture findings (130). At any decision level, one must sacrifice sensitivity for specificity and vice versa. Herein lies the major flaw of (laboratory) diagnosis, in that the tests are not sensitive and specific at the same time. Looking at multiple laboratory tests improves the predictive value somewhat, but the

Prevalence of disease (%)	Predictive value (%)
1	16.1
2	27.9
5	50.0
10	67.9
15	77.0
20	82.6
25	86.4
50	95.0

Table IV. Predictive value as a function of disease prevalence for a laboratory test with 95% sensitivity and 95% specificity. (Table IV and Fig. 7 (Legend slightly modified) are reprinted with permission from RS Galen, Blood Cells 1980; Ref 130; Springer Verlag, Heidelberg)

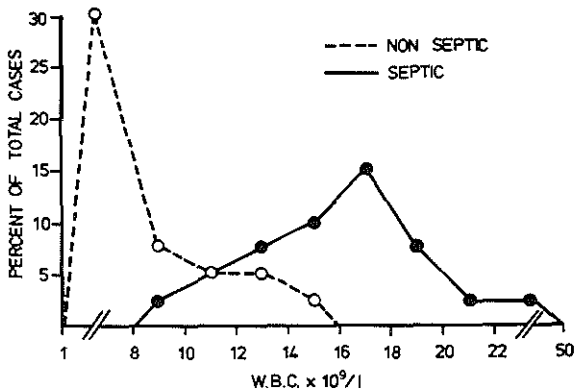

Fig 7. Overlapping distributions of WBC in septic and nonseptic patients (prevalence 50%). The selection of the upper limit of normal (decision level) determines the sensitivity, the specificity and the predictive value of a positive result of wbc-counts as follows:

Decision level	Sensitivity (%)	Specificity (%)	Predictive value (%)
$8x10^9/l$	100	60	71.4
10	95	75	79.2
12	85	85	85.0
14	70	95	93.3
16	50	100	100.0

fundamental trade-off between sensitivity and specificity always remains (see S 4.2.4).

4.2.4. Multivariate analysis.

Solberg (131) was the first to illustrate graphically the benificial effect of multivariate analysis. Fig. 8 is a diagrammatic representation of a multivariate analysis with two variables. By combining the two tests we may be able to effect a greater degree of discrimination than could be achieved by using either of the two tests considered separately. It is possible to apply the same principle to more tests, although this is difficult to visualize. This approach has proven valuable in defining the combination of tests which will maximize the probability of a given diagnosis.

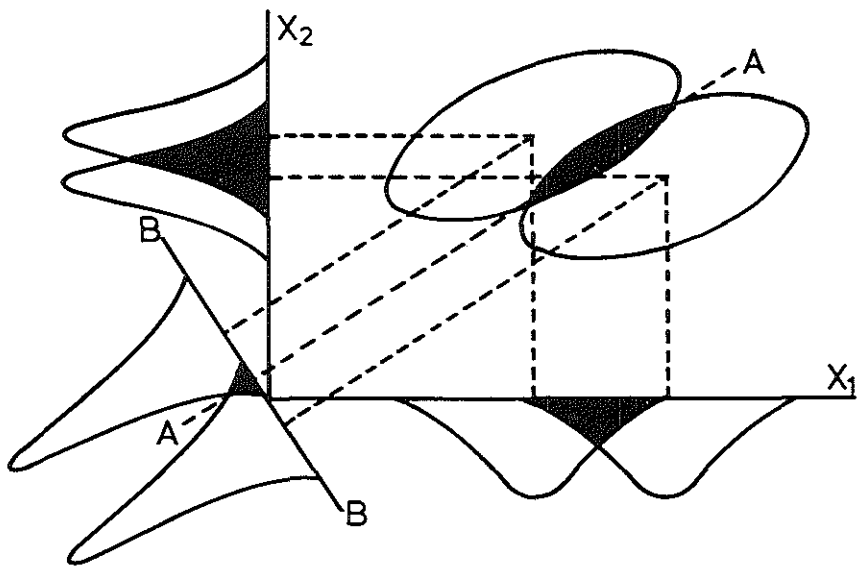

Fig. 8. Discrimination between two groups in the bivariate case. (From Solberg HE, Scand J Clin Lab Invest 1975; 35: 705-12, with permission; Ref 131)

Unfortunately, its diagnostic application is difficult for several reasons (132). Beck et

al. (133) applied (computer assisted) multivariate analysis to iron deficiency anaemia to generate an efficient sequence of diagnostic laboratory tests. They constructed a three step diagnostic system: serum ferritin level and MCV as a screen in all patients, followed by serum iron and iron binding capacity in some patients and by erythrocyte sedimentation rate in a few patients. When compared to bone marrow iron stores, this system was found to have 96 per cent predictive value.

5. Aspects of cost-effectiveness and QA

In the present economy, much concern has been concentrated on the relationship between cost and medical efficacy of laboratory tests. It has been clearly shown that test ordering is fundamentally developed during internships and residential training (134). It is one of the paradoxes of contemporary medicine that there is a vague, but definite, malaise surrounding laboratory diagnosis at a time of unparalleled accomplishment in techniques and equipment. Too many available choices, especially if imperfectly understood, create confusion in the chooser. Laboratories have very little control of ordering and sequencing of the requisitions. Hovind (134) describes the reality of the present situation as follows:"Both in clinical and in laboratory practice, the junior doctor armed with the best intentions enters the laboratory super-market, whose shelves are increasingly filled with exotic and expensive tests and orders tests at will, regardless of cost rationale, and consideration of the patient". Galen and Gambino (135) formulate the situation as follows:"In the use of laboratory tests the physician does not sense any limitation. He considers the laboratory an infinite resource. A simple, effortless, painless stroke of the pen is all that is

required to command its use. It is not the physician's time that is used. It is not the physician's money that is consumed. It is not the physician's assistants who do the work".

In the direct clinical care of the patient one of the objectives of QA is to reduce health care costs through appropriate test requisitions and avoidance of the necessity to repeat tests. There is little doubt that one of the most important ways of dealing with the "data explosion" is to further the communication and co-operation between the laboratory and the clinician. Much of the data might not be effectively converted into information of medical value. It is the significance that is assigned to data (S 4.2) which converts them to information (10).

An example of how to possibly tackle the problem has been given by the American Medical Association since 1975, by publishing a series of publications "toward optimal laboratory use" in the JAMA (136). One of the most recent contributions in this series deals with the considerable misuse of tests in a teaching hospital, under the intriguing title "Ready! Fire!.....Aim! An inquiry into Laboratory test ordering (137) (cf Fig. 1)

Advances in technology have caused the unit cost of laboratory tests to decrease, but the variety of tests and gross expenditure for the total number of tests requested has increased drastically. This technology has also provided an alternative to selective testing in the form of multiparameter (panel) testing. The question is sometimes raised whether panel testing in case of a simple selective (e.g. Hb) request adds to the ever increasing expenditure on laboratory analyses. Lehman et al. (138) have shown in a comparable chemistry situation that panel testing resulted in substantially lower costs and fewer return visits to the laboratory than either selective or mixed

(combination of selective and panel) testing. Moreover, in every day practice IQC in multiparameter instruments is less time-consuming and thus easier to perform.

Serious studies are necessary as to whether the initial requisition by all physicians of standardized optimal protocols (chosen after careful consideration and literature studies) rather than the individual requisitions per patient and per physician for the diagnosis of a presumed disease, could fundamentally contribute to cost control without impairing the quality of the requisition. The three-step diagnostic system for iron deficiency anaemia described by Beck et al. (133) or the improved classification of anaemias based on MCV, RDW and rbc-histo-grams (83) might be candidates for such a standardized, optimal protocol.

Chapter III

BASIC PRINCIPLES AND PROBLEMS OF HAEMOCYTOMETRY

1. Introduction

The advent and widespread acceptance of flow haemocytometers (hcs) has markedly improved the precision of haemocytometry (hcy). Accuracy on the other hand is still a source of major concern. This is brought about by several factors. In this review we shall discuss the basic principles and problems of hcy. It will be shown that some of the classical manual techniques (microHct;Hb) still basically contribute to resolving accuracy problems.

2. Principles and problems of manual haemocytometry

2.1. Cell counting chambers

For two reasons we shall not indulge in extensive descriptions of and comments on counting chambers (c.c.):

a. they have gradually been replaced by flow haemocytometers (hcs);

b. many detailed descriptions and critical reviews on c.c. have been published (1-5).

Nonetheless, some remarks may be appropriate. Although counting precision and accuracy have always been a major source of concern, c.c. theoretically provide inherent primary calibration possibilities. Some authors (1,2) believe that this goal of absolute counting can be achieved by improving the c.c. as well as the counting technique. There are, however, major sources of error remaining in chamber counting (1-5), such as variable chamber depth mainly due to the positioning and bowing of the cover glass (this problem being reasonably well resolved), systematic differences existing between the results obtained by even experienced technicians (5) as

well as problems inherent in the counting of too few cells due to (near) Poisson distribution statistics (1). For a full account on the subject we refer to the above-mentioned references (1-5).

This implies that c.c. seem not (yet?) to be suitable for calibration of flow hcs, not even by (inter)national institutes for metrology, and despite modern possibilities, such as video display monitoring or photographing of the c.c., and accurate checking of the chamber depth by micrometers or interference comparators (1,2,5). This is substantiated by the fact that recent literature (4,6,7-9) and flow hc Manuals (10) generally describe cell counting calibration by comparison with values found in digital flow hcs (see S 3.2.2.) (see also Ch II).

2.2. Manual haemoglobinometry

For the determination of haemoglobin (Hb), the spectrophotometric method using cyanhaemiglobin (HiCN) has been accepted worldwide, and recommended by the ICSH (11-12). Comprehensive, detailed reviews (5,11-14) on the theory of and the experience with this method have recently been published. Although it is not our intention to repeat this information, again, it seems appropriate to comment on this method (11-18).

Indeed, the HiCN-method has - as compared to other known methods - several significant advantages:

(a) only a single reaction has to be used;
(b) all Hb species and derivatives are monitored, i.e. deoxy-Hb, oxy-Hb, carboxy-Hb, fetal Hb and (at least in part) sulph-Hb;
(c) the stable reaction product HiCN has a broad absorption maximum at 540 nm;
(d) the Lambert-Beer law is valid within a wide range of absorption;

(e) stable standard solutions can be prepared either from crystalline Hb or from washed rbc and they can be shipped without severe problems; and
(f) since HiCN is a stable derivative of Hb, measurements of the absorbance can be performed some min. or even some days after the addition of blood to the reaction medium without significant alteration of the values obtained.

Nevertheless, the HiCN-method has also some disadvantages which cannot be neglected:
(a) since the HiCN-reagent contains cyanide, it is toxic and therefore has to be handled very carefully;
(b) the reaction solution is light-labile (even in the dark, it is not stable for more than 6 months);
(c) HiCN-solutions from patients with marked hypergammaglobulinaemia or paraproteinaemia may be turbid and must be clarified by centrifugation for 15 min (Turbidity can be prevented by the addition of NaCl, by increasing the conc of the phosphate buffer, or by adding a drop of ammonium solution (17));
(d) the conc of the reaction components, especially those of cyanide and the buffer have to be chosen and kept constant very carefully;
(e) standardisation of the method is based on purified HiCN-solutions, the quality of which is controlled only indirectly by spectrophotometry (direct iron analysis is not possible because of the presence of $K_3[Fe(CN)_6]$ in these standard solutions); and
(f) the reaction times of the different Hb species and derivatives differ markedly (3-5 min for Hb) and at least in the case of carboxy-Hb the time for conversion into HiCN (90-120 min) is too long for rapid clinical use, giving rise to too high values (up to 6%; (14) (This period can be decreased to 15 min by increasing the ferricyanide conc fivefold (15)

or to 3-5 min by heating the reaction mixture to 56°C) (15). It should be realized that carboxy-Hb values for smokers (particularly those who inhale cigar smoke) may exceed 20%! (18). (Surprisingly, a negligible effect of carboxy-Hb on Coulter Model S readings (conversion time of some 20 secs!) was found (18). The broad-band-pass filter (495-555 nm) of the Coulter S and/or the reagents used may possibly explain the phenomenon. (11,18)).

Quite recently (15,16) a new method for the rapid and accurate measurement of Hb has been developed as an alternative to the conventional HiCN-method. This method is based on the conversion of all haeme, Hb and haemiglobin species into a stable end product by an alkaline solution of a non-ionic detergent ("AHD reagent"). The reaction product, designated as alkaline haematin D-575, is extremely stable and shows a characteristic absorption peak at 575 nm. As compared to the HiCN-method, the determination of Hb by alkaline haematin D-575 offers several advantages such as:

(a) extreme stability of the AHD reagent and the conversion product;
(b) decreased conversion time of all Hb species into the end product;
(c) decreased amounts of plasma and cell errors, and errors caused by delayed conversion of carboxy- and fetal haemoglobins; and
(d) standardisation by a primary standard (purified crystalline chlorohaemin).

This new method certainly deserves further studies.

Finally, quite sensitive Hb-determinations in plasma and serum have recently been described, obviating the potentially carcinogenic benzidine (19,20).

2.3. Centrifugal haematocrit (comparison with flow cytometric haematocrit)

2.3.1. Macro versus microhaematocrit

The haematocrit (Hct) is the ratio (expressed as a fraction or as a percentage) of red blood cell volume and whole blood volume in a particular whole blood specimen. Centrifugal (cf) Hct can be determined either by a macromethod or by a micromethod (21,22). ICSH have recently published "Recommended methods for the determination of packed cell volume" (21,23) or cf Hct. Although ICSH consider the terms Hct-value (Hct-ratio) and packed cell volume as synonymous, we prefer the term "haematocrit" (although the term "haematocrit" was originally defined as the tube in which blood is centrifuged or the centrifuge used for this purpose (7)), as, strictly speaking, the term packed cell volume is a misnomer in that it represents a volume ratio rather than a volume. (the correct terms relative or fractional packed cell volume are sometimes used in the literature (24,33)). In 1980 ICSH also published "Recommendation for reference method for determination by centrifugation of packed cell volume of blood" (25). This method recommends the macromethod as a ref method for the Hct determination, mainly on two grounds:
a. "the potential lack of uniformity of the bore of many makes of microHct tubes" and
b. even in the micromethod the amount of "trapped plasma may account for about 3% of the apparent red cell column in normal blood and even more in certain abnormal conditions" and "there is, therefore, no intrinsic merit in selecting a centrifugation method that has a relatively low plasma trapping correction". Radioactively labelled human serum albumin has been chosen as the indicator for determining the trapping correction since it is a well-established method (26,27).

However, this macromethod is - like most ref methods - highly unpracticable for routine (calibration) purposes. Fortunately, recent studies have largely refuted the above-mentioned arguments against the use of the micromethod, at least as a practical calibration method (28,29):

a. It is recognized that some commercial firms put high-quality microHct-tubes on the market (27). Acceptable uniformity in bore (<2%) can be indirectly assessed by determining that the intra-assay CV of the microHct of blood samples meets certain criteria. Depending on the bore and the thickness of the wall of the tube as well as on the skill of sealing and melting the tube end, CV's varying from 0.2 to 0.8% can be achieved (8,27). Even tapered tubes can be successfully used, provided appropriate corrections are applied (27).

b. Recent re-assessment of the amount of trapped plasma by Pearson and Guthrie (31) showed a degree of trapping amounting to 1.53 +/- 0.33 (2 SD) % for normal blood (n=25), provided the centrifugation has been carried out at approximately 13,000xg (at the buffy-coat interface) for ten min (in contrast to ICSH which recommend only five min). A further reduction of trapping is not possible even in an ultracentrifuge or with an excess of spinning time, if heating by air friction is avoided (8). /The non-equivalence of flow hcy and cf (predominantly macro-)Hct's of rbc, serially diluted in their own plasma, as described by Fairbanks (32), is also (mainly) attributable to too high and variable percentages of trapped plasma in the (non-corrected) centrifugation methods used in that study./

2.3.2. Centrifugal haematocrit of normal blood.

The corrected microHct of fresh, normal human blood approximates the true Hct value, taking into account the above mentioned and the following conditions:

Fully oxygenated blood should be used as the Hct decreases by about 3% (1 Hct%) in oxygenating blood due to Gibbs-Donnan equilibrium changes (21,22,33). Moreover, di-K-EDTA in excess of 2 mg/ml blood will cause cell shrinkage (25) and so does the use of tri-K-EDTA instead of di-K-EDTA (27, 30).

For all practical purposes, the mean of microHct values of, say, ten fresh, normal blood specimens is a very reliable calibrator for flow hcy instruments, provided 1.5% allowance for trapped plasma is made (8). In our experience, this procedure can be considered as an excellent practical calibration method (28). Likewise, NCCLS (29) recommend their issued "approved standard procedure for determination of packed cell volume by the microhematocrit method" as an intermedium for the calibration materials for automated, multiparameter hematology analyzers (29).

2.3.3. Centrifugal haematocrit of pathological blood

Pearson and Guthrie (31) found that the trapped plasma results for 127 specimens (including 25 normal specimens) ranged from 1.18% to 2.25% (mean 1.61%; SD=0.213), provided the MicroHct was performed at 13,000 x g for ten min. They found only six specimens which had trapped plasma volumes equal to or slightly greater than 2%. Of these, three were from patients with marked microcytic hypochromic changes due to iron deficiency anaemia. The

remaining three specimens were from patients with abnormal haemoglobins. In another paper (34) they describe their important findings of Hct-values in 17 polycythaemia patients: whereas the microHct's gave correct values, the amount of trapped plasma varying between 1.2 and 1.8% (mean 1.55%), the Hct's were severely underestimated by Coulter's Model S and S-Plus instruments (6.4 and 7.4% at an MCH of 1.25 fmol, respectively). This was earlier described by Penn et al (35) and Nosanchuk (36). (cf S 3.4.3.3)

Inaccuracies in the Hct's (MCV's), as measured by Coulter S, S-Plus and ELT-8 instruments, of pathological rbc due to changes in deformability and ensuing shape factor (S 3.4.3.3.) and due to changes in refractive index, respectively, lead to discrepancies with the corrected microHct method: Flow hcy Hct's are too high at high, "true" MCHC (i.e. MCHC calculated via a corrected cf Hct) and too low at low, true MCHC (37-39), although in one study (37) Coulter S (Plus) Hct's agreed with microHct's at low MCHC values.

Quite variable discrepancies among flow hcy Hct's and between flow hcy Hct's and cf Hct's occur in normal rbc, in a variety of disease states producing hyperosmolar plasma, namely hyperglycaemia and uraemia. An extensive background discussion on the underlying osmotic matrix effects giving rise to the spuriously elevated flow hcy Hct's has recently been given (40) (cf Ch II, S 3.1.2.1.).

These findings have important implications for the diagnostic value of the MCHC, being the ratio of Hb and Hct. Whereas the MCHC, determined with the macro-Hct-method, decreases in iron deficiency due to increasing plasma trapping, MCHC generally remains constant when determined with the appropriately performed microHct. MCHC will also be constant in flow hcy instruments, the more so as flow hcy MCHC

generally is too high at low cf MCHC (true MCHC) and too low at high cf MCHC (37-39). Consequently, since MCHC, as measured with modern techniques, is generally constant, its diagnostic value is negligible (41-44), whereas it is very useful for IQC purposes (45). (cf Ch II, Fig. 6, p.55)

2.3.4. Centrifugal haematocrit of artificial blood

Artificial blood is designed primarily for use in flow hcs. Hct's produced in these instruments generally do not match those found by the cf (micro)Hct method. Consequently, this impedes the microHct of artificial blood to be used for calibration purposes.

MicroHct discrepancies may occur in two ways:

a. MicroHct values higher than flow hcy values. This is mainly caused by the fact that the preserved or fixed cells do not pack evenly under cf force due to decreased deformability, resulting in a falsely high cf Hct (6). On the other hand flow hcy Hct values may be too high, albeit less pronounced, due to the same decreased deformability and its resulting increased shape factor of the red blood cells (8,39) (see S 3.4.3.3.). An extreme example is given by the highly discordant Hct results of "Stabicell" (formaldehyde-fixed rbc)-suspensions, their microHct values being virtually twice as high as the Coulter Model S Hct-values (5).

b. MicroHct values lower than flow hcy values. This is clearly demonstrated in Table I, showing the discordant values of Hyland's 8 parameter control material (unfixed rbc) (46). This representative example shows a ratio of MicroHct to Coulter-Hct of about 75%. We also measured the Hct of the normal control with the very accurate 131I-albumin method of England

c.s. (26) and found 50.2%. This strongly suggests that the MicroHct is too low. The most plausible explanation for the phenomenon is given by Halbhuber et al. (47). Using the 131I-albumin method they showed 20% lower values for spun Hct's than for electronic Hct's in rbc in artificial media compared to these in autologous plasma. They showed that this was due to a medium dependent permeability of the rbc membranes, causing compression of rbc with concomitant H+ and H2O efflux (47).

Finally, the effect of rbc storage lesion on the Hct, estimated by the ICSH ref method, the micromethod, and the Coulter counter S, has been recently described (48).

Table I.

Haematocrit values of Hyland's normal and abnormal 8 parameter controls (No 814 H2) with manual and flow cytometric techniques. Values in parentheses indicate the number of measurements.

Control	CS(a,c)	ELT-8(b,c)	Mhct	131I-Alb	Mhct/CS
Low	27.8 (5)	25.2 (2)	20.3 (2)		~ 75%
Normal	50.1 (2)	46.8 (1)	37.5 (2)	50.2 (2)	~ 73%
High	60.0 (1)	55.6 (1)	46.5 (2)		~ 78%

a). CS = Coulter Model S
b). Ortho Diagnostics, Westwood, Ma 02090, USA
c). Values in conformity with stated values

3. Principles and problems of flow haemocytometry

3.1. Introduction

The basic concept of flow hcy is that blood cells are made to flow at high speed through a sensing region where electrical or optical cell properties are measured. The cells are

suspended in a carrier fluid, usually an isotonic buffer, which is used to make the proper dilutions and to transport them through the measuring region.

A knowledge of the principles governing this fluid flow is important for the understanding of the events that occur during measurements, and the effect of the flow on the data that is obtained. Important improvements in fluidics and electronics, such as hydrodynamic focusing, sweep flow and pulse editing, offering unsurpassed cell resolution and enabling simultaneous counting of plt and rbc, shall be described (S 3.3.2.).

3.2. Classifications of flow haemocytometry instruments

Coulter's instruments are the first and foremost in electrical ("aperture-impedance" or "resistive-particle") flow hcy (50,51) (S 3.4.). Optical (light-scattering) flow hcs are represented by Technicon's H-6000 series and Ortho's ELT-instruments (S 3.5).

In the measuring region the cell conc is determined either directly with a digital method by counting the number of cells in a given volume (e.g. Coulter A, ZBI etc), or indirectly with the analogue method by counting the number of cells per unit time (routine flow hcs: Coulter S-Plus series, ELT series, H 6000(C) (8). Common to both counting principles is the discriminator for threshold setting and the transducer, a device that generates electrical pulses when cells pass the electrical or optical sensing zone.

3.2.1. Analogue instruments (8,49-58)

In analogue instruments, the number of pulses per unit time, the counting rate, is measured. This rate is converted into a

proportional but otherwise arbitrary voltage which can continuously be read. An adjustable amplifier is employed to amplify the voltage into an appropriate value for display of the cell conc. This displayed value has to be calibrated to the assigned value of the calibrator. This means that the zero intercept and the slope of the amplifier have to be adjusted by means of a particle-free sample and a ref suspension with a well-defined cell conc. (Ch II, S 3).

The major routine flow hcs are analogue instruments. We shall not describe them but rather refer to their Manuals and descriptions and/or evaluations in the literature: Coulter's Model S-Plus series (49-53), Ortho's ELT instruments (51,52,54,55) and Technicon's H6000 series (51,56-58)

3.2.2. Digital instruments (5,8,59,70)

With the digital method, the cell conc is determined directly by counting the number of cells in a given volume. This volume is a calibrated liquid column between a start and a stop sensor (5). The accuracy of these instruments is mainly dependent on the accuracies of this measuring volume and the counting device as well as of the cell dilutions, threshold settings and coincidence and background corrections (5) (See S 3.4.2.) Examples of these digital instruments are the Analys 134, Contraves 800, Coulter Models DN, ZB and ZF, Sysmex CC 108, 110 and 800 (60) and the Ultra Logic 800 (59). Detailed accuracy studies on these instruments are currently being performed by Helleman (59) (See S 3.4.2.) Digital counters are often used for primary calibration (cf. S 2.1 and Ch II, S 3.6). Important remarks and recommendations on accuracy in cell counting for use in calibration, are given by Thom (8) and Harfield

(9), using a Coulter counter of the ZB-type.

3.3. Hydrodynamic properties of flow haemocytometry instruments

3.3.1. Flow of fluids in the sensing (impedance) apertures (Courtesy V.Kachel and M.L.Mendelsohn, Refs 61,70).

Flow in tubes is either laminar or turbulent. In flow hcy instruments, the suspending medium must transport the cells along well-defined paths through the sensing region. Thus, since accurate cell transport trajectories are required, laminar flow conditions must be established and turbulence avoided. For flow in tubes, this requires that the Reynolds number (Rc) be below the critical value of 2300. That is,

$$vdp/z < Rc = 2300$$

where v is the average flow velocity, d is the tube diameter, p is the fluid density, and z is the fluid viscosity coefficient. For water in a 100 um tube, this leads to laminar flow below 23 m/sec. Above this velocity, turbulence tends to occur. The possibility of turbulence increases with distance along a tube or with the presence of surface irregularities. Just inside the tube entry of a laminar flow system, the velocity profile of the fluid is flat, with a sharp drop to zero very close to the wall (i.e. the boundary layer). There is a gradual transition from this distribution to a parabolic distribution with increasing distance into the tube. In the parabolic velocity distribution, the maximum velocity is on the axis, the velocity at the wall is zero, and the average velocity is one-half the axial

velocity. For water flowing in a 100 um-diameter tube at the critical velocity of 23 m/sec, little progress toward the parabolic velocity distribution occurs in the first few hundred micrometers into the tube; hence, practical flow channels usually found in flow systems generate essentially flat velocity distributions, with a core region of linear velocity distribution. The radius of the core region is a function of the Reynolds number (usually in the order of 300) and the distance from the inlet of the aperture.

3.3.2. Basic fluidic (and electronic) improvements enabling high-resolution cell analyses.

3.3.2.1. Hydrodynamic focusing

Both the ELT and the H-6000 (optical) instruments apply a technique called hydrodynamic focusing, a key fluidic improvement. In these flow hcs a particle-free sheath fluid is used to constrain the cells to be measured in the center of the sensing region, eliminating artifacts caused by pulses from off-axis cells (see below), thus greatly improving cell resolution (62). The cells are forced into a single-file array and intercept the light beam, which is focused to a very small sensing zone. In the ELT instruments the passing cells scatter the laser light and the low angle forward light intensity data is processed (54-55). In the H-6000 a dual detection system in the peroxidase channel measures light scatter and light absorption due to peroxidase stain (58). Additional claimed benefits of sheath fluid technology include virtual elimination of clogging and minimal cell transit time, which reduces coincidence error and increases analysis rate (62).(cf S 3.5)

In "aperture-impedance" or "particle-resistive" (Coulter-type) counters (39,49,60-67), cells in fluid suspension flow through a small orifice or aperture, causing a change in the electrical resistance across the aperture, as they are essentially non-conductors of electricity. This change produces a voltage pulse, the signal height of which is not simply proportional to the volume of the cell, but rather to the volume of the electrolyte displaced within the sensing zone, and, more importantly, to the changes of electrical lines of force, the so-called "electrical shadow" (49,67). In other words, the pulse height is also dependant on the orientation and shape of the sensed cell (see S 3.4.3.2.) as well as on the current and fluid densities along the path taken by the cell through the aperture. Because the electrical and hydrodynamic fields along and across the aperture are not uniform, even identical (latex) spheres having different trajectories give voltage pulses of different shape and duration. Orientation and shape of (flexible) cells are altered near the wall by the unbalanced shear forces there. Consequently, it will be obvious that the residence time in the aperture for a cell travelling near the wall will be longer than that for a cell passing through the center, because of the smaller fluid velocity near the wall (boundary layer, see S 3.3.1.). Consequently, duration of the signal created by a cell travelling near the wall will be correspondingly greater.

Ejection of cells from the tip of a focusing tube placed approximately 1 mm in front of the aperture, has repeatedly been shown (39,61-67,70) to align the cells in axial aperture flow. This procedure is also called hydrodynamic focusing. It produces dramatic improvement in pulse resolution by preventing aberrant pulse shapes and artifactual pulse

heights, thus enabling simultaneous rbc and plt counting and sizing. However, probably for pratical reasons (e.g. analysis speed, technical problems) this focusing technique is not applied in routine flow hcy. Instead, other procedures such as pulse editing and sweep flow are applied to achieve sufficient cell resolution.

3.3.2.2. Nonaxial cell flow and pulse editing (9,49,53,62-64,66-68)

Since Coulter-type counters generally do not have hydrodynamic focusing (49,68), pulses are allowed to be sensed from cells travelling through various parts of the sensing apertures. As by choosing optimal aperture dimensions only a fairly small portion of the cells will be nonaxial, there will be a relatively small artifactual right skew of the cell distributions. In routine flow hcy, electronic circuits edit rbc and wbc pulses to exclude those produced by nonaxial cells (10). The edit circuits select only those pulses coming from one by one central passage through the sensing zone. Such pulses have width to height ratios for single cells within tightly definable limits (53).

3.3.2.3. Sweep flow (9,49,61,62,67,70)

At the outlet of tubes (apertures) flow transition from a small-diameter tube to a large-diameter tube occurs. The divergence angle at the widening tube is not allowed to exceed some 8^{o} to prevent flow separation with formation of turbulence. So-called fluid eddy currents and a backflow of part of the fluid and cells ejected by the aperture occur, producing distorting pulses (70). In modern eight-parameter Coulter counters the so-called sweep flow (49), a steady stream of diluent

that flows behind the rbc apertures during the sensing period, prevents cells from re-entering the sensing zone and being counted as plt. The sweep flow technique provides many of the advantages of hydrodynamic focusing, enabling the simultaneous counting and sizing of rbc and plt. (49,67)

3.4. Electrical (aperture-impedance) counting and sizing (Coulter principle)

3.4.1. Introductory (Courtesy V.Kachel and M.L.Mendelsohn, Ref 70)

The issuance of a patent for an electrical resistance technique for high-speed cell counting to W.H.Coulter made the year 1953

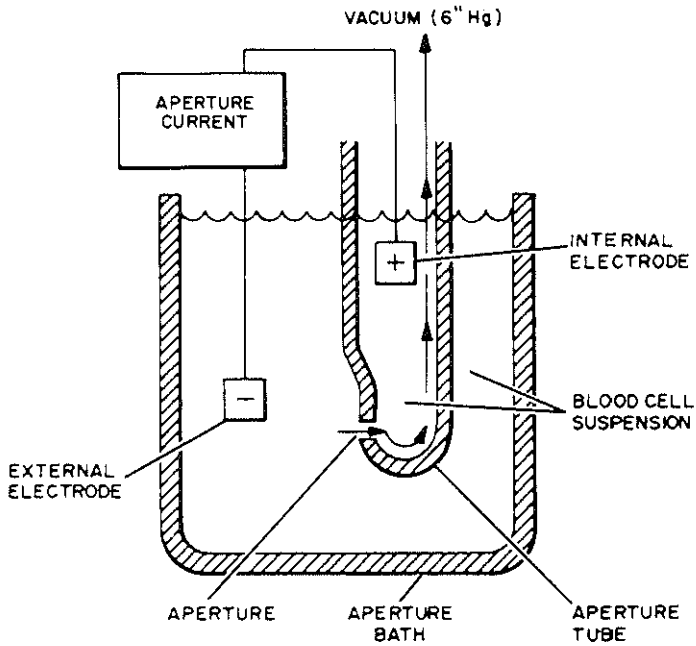

Fig 1. Coulter Method of Counting and Sizing (Reprinted with permission of Coulter Electronics Ltd, Luton, UK)

a landmark in the development of flow hcy systems. Over the next few years this brilliant invention was developed commercially into the "Coulter Counter", now well known for its successful application in clinical haem. laboratories (70). Coulter's idea is easy to comprehend but its simplicity is deceptive. Imagine a dilute suspension of cells in saline being drawn through a narrow aperture (50-100 um in diameter typically). At the same time a constant current, i, flows through the aperture. (Fig. 1) (70)

Since cells are poor electrical conductors due to membrane resistivity, a cell traversing the aperture causes the electrical resistance, R, to be momentarily increased, $\triangle$ R, thus generating a voltage pulse of amplitude i x $\triangle$ R. These electrical pulses can easily be amplified and counted; at the same time the volume of cell suspension flowing through the aperture is measured, yielding the number of cells per milliliter of suspension. This rather simple measurement turns out to be extremely useful in clinical haematology and biological research. It is widely used for counting blood cells. (70)

In addition, by measuring the voltage pulse it is possible to determine the cell size and thus obtain size distributions of cell populations - important information very hard to get any other way. Although easy to verify qualitatively, the quantitative relationship between cell volume and pulse height turns out to be complex, especially for human rbc around which was centered most of the early work and controversy. We now have a much clearer understanding of the important variables such as cell shape and orientation, shape changes under the hydrodynamic forces in the aperture, the cell trajectory through the aperture, the electrical field distribution in and near the aperture, the aperture dimensions, coincidence

effects, and electronic instrumentation effects. (70)

3.4.2. Blood cell counting

3.4.2.1. Counting accuracy with special reference to red blood cell counting

The difference between analogue and digital counting has already been described in S 3.2.

In Ch II, Fig. 2 and S 3.1.1. we already mentioned the substantial variation in the mean counts of the same blood specimen produced by different instruments, as described by Thom (8). Similarly, Helleman (59) found differences in results varying from 3.5 to approx. 5%, when comparing a number of digital instruments. Harfield (9) describes a scatter of values around the average ranging from -11.8 to +16.4% from 10 individual laboratories using several different Coulter counters.

Accuracy of count results is dependent on the correctness of the following items (5,8,11):

a. the counting volume
b. the counting device
c. the discriminator setting
d. coincidence correction
e. background pulses correction
f. blood sample dilution factor
g. integrity of the blood sample (absence of count loss due to lysis and/or adherence of cells to the wall of the container and/or due to cell clumping; absence of count gain due to contamination)
h. absence of other interfering causes, such as leaks, plasticizers, abnormal cells etc (11).

We shall briefly discuss some of these

accuracy items.

a. The counting volume. Digital instruments have a controlled displaced liquid volume (5,67). In a Coulter Counter this displaced volume is factory calibrated to at least 0.25% of the stated volume by procedures traceable to the U.S National Bureau of Standards (67). In analogue instruments accuracy is highly dependent on the accuracy of the calibration (Ch II).

b. The counting device. Proper electronic instrumentation is essential for accurate counting and sizing. The counting device can be tested by aid of the voltage pulses generated by a Particle Simulating Instrument (PSI) (5) or other pulse generator (11,70), such as those built in the latest Coulter Models to perform "ramp" and precision tests. For other tests necessary for optimal performance we refer to the various instruments' Manuals (See also Ch II, S 3.1.1.).

c. The discriminator setting selects wanted from unwanted pulses. This setting is particularly important with regard to cell counting accuracy, as improper setting causes count loss or count gain. Moreover, the setting accounts for the degree of coincidence loss (see below). Last but not least, accurate setting is vital to the appropriate differentiation of cell types e.g. plt and microcytic rbc or lymphocytes and granulocytes. Helleman (5) comments on the calibration of the setting of Coulter type counters. By aid of the PSI the necessity of recalibration can be explored and zero setting and linearity of the discriminator can be tested; moreover it is possible to compare the response to pulses of two similar counters. Provided zero settings

and linearity of the discriminator are correct, the value of the "critical discriminator level" can be calculated from the relative change of the value of the mode or the counting plateau of the frequency distribution curve of a suspension of stable particles of the relevant size (e.g. approx. 35 fl. to discriminate plt from microcytic rbc) (5).

d. Coincidence correction and
f. blood sample dilution factor (5,70).

Coincidence is the phenomenon occurring when at the same moment two or more particles appear in the sensing area of the detecting device, and when two or more pulses follow each other so closely, that they cannot be discriminated by the discriminator device, and also when two or more count pulses follow each other at such short time intervals that the counter is not able to count these pulses separately (5). If the identical cells move through the aperture as if stuck together, a double-sized pulse results and the two cells are incorrectly measured as one cell of double volume. If they move through the aperture in tandem sequence then a double-humped pulse results and again a false value is recorded. The latter effect can be recognized electronically and rejected. (70)

Coincidence error is dependent on the length of the sensing zone and on the conc, flow rate and size of the particles (61,67,70). For a nominal rbc-count of 5×10^{12} rbc/l counted at a dilution of 1 in 50,000, the error will be 12% in an aperture which is 100 um in length; of the 12% loss, 10% is the result of geometric cell coincidence and 2% is caused by electrical time constants (the time constant of the amplifier will affect the ability of the detector to recover from a pulse in time to respond to a following one).(67) Although the magnitude of the error diminishes with decreasing cell conc, the error cannot be brought down by increasing the dilution of the

specimen from 1 in 50,000 to, say 1 in 1,000,000. Such an approach is undesirable because dilutions as great as 1 in 1,000,000 pose problems of volumetric accuracy (8,67), they downgrade precision from 0.5% to 2% and, more importantly, these high dilutions generally are not stable, because of the adhesion of cells to the wall of the tube or the container (8,11). There is a loss depending on the properties of the material, the shape and size of the cells and the surface properties of the container (8,11). Adhesion could even be increased by agitation of the filled container on a roller-mixer (8).

A more effective alternative is to replace the standard aperture with one that has a diameter of 0.030 mm and a length of 0.050 mm (67,70). The reduction of the sensing zone volume achieved by this arrangement results in a reduction of coincidence error by a factor of at least 20 or to a level of 0.6% for normal blood diluted 1 in 50,000 (67). Furthermore, the error is now virtually a linear function of count and its magnitude can be confirmed by performing an additional count at a dilution of 1 in 100,000 (67). This simple approach to extracting the full-potential performance from a single counter is not without penalty, since the time it takes liquid to pass through the smaller aperture is considerably greater than in standard (routine) counters and the chance of aperture obstruction is increased (67). It will be clear that routine cell counting instruments are without exception optimized in speed by sacrificing accuracy (8). However, when a counter is to be used in a ref method, the coincidence error should be removed rather than corrected after the fact (67).

For a mathematical elaboration on coincidence theory, we refer to Helleman (5,59).

3.4.2.2. Platelet and white blood cell counting

Thanks to the various fluidic and electronic improvements described in S 3.3.2, plt can be counted simultaneously with rbc (61-70). Instruments not equipped with these high-resolution devices, require prior separation of plt from rbc, usually by differential centrifugation, yielding so-called platelet-rich plasma (11).

A particularly important source of error in plt counting due to anticoagulant-induced pseudothrombocytopenia has already been discussed on in Ch II, S 3.1.2.1.

All methods used for wbc counting necessitate a haemolysing reagent to eliminate the rbc which exceed the wbc numbers in normal blood by approx. 1000:1. Wbc are counted in separate counting apertures, the dimensions of which are different from those of rbc apertures (10). It is a matter of course that incomplete rbc lysing will lead to falsely high wbc counts. On the other hand, improper discriminator setting will generally cause count loss. As wbc counting and sizing are closely connected, we refer for further details to S 3.4.3.6.

3.4.3. Blood cell sizing

> ... and after the biologist and the chemist had completed their descriptions of the horse, the physicist began: "Now let us first assume that the horse is a sphere" ...
>
> Kerker et al (71)

3.4.3.1. Primer of aperture-impedance theory

Kachel (70) shows that the electrical signal from a Coulter sensor depends on cell and aperture parameters as follows

$$\triangle U \sim Vpif/r^4$$

where
$\triangle U$ = amplitude of voltage pulse
V = particle volume
p = electrolyte resistivity
i = aperture current
f = cell shape factor
r = aperture radius

The equation applies to "long" apertures (length-diameter ratio between 1 and, say, 2) such as in the Model S-Plus II, where rbc apertures length and diameter are 60 and 50 um, respectively. As can be seen, the output voltage is strongly dependent on the aperture radius r, showing the importance of small apertures for measuring small objects

While pulse height is essentially proportional to the electrolyte resistivity p, particle resistivity has very little effect on the voltage response, unless it is quite close to the resistivity of the liquid. If the particle resistivity changes from one million to 100 times that of the electrolyte, there is less than 1% change in the response (67,70).

The distribution of current density i in and near the aperture is important. The equation shows that the voltage pulse is also proportional to i, which is dependent on the position in the short aperture commonly used, especially near the edges where the current density is maximal. As a result, pulse shape and amplitude vary with the cell trajectory, causing inaccurate cell-volume distributions.

As we have seen, this problem can be partly solved by electronic editing or, preferably, by hydrodynamic focusing. (S 3.3.2).

Finally, $\triangle$ U is linearly proportional to f, the cell shape factor. This is dealt with in the next Section

3.4.3.2. The shape factor.

In an aperture current field the lines of force around a rigid sphere follow a fusiform shape, creating an electrical "shadow" in the field that is very nearly 1.5 times the volume of the sphere itself. It is the volume of this "shadow" that determines the height of the pulse (49,63,67,70). A sphere is said to have a shape factor 1.5. Particles with other shapes produce more complex shadows, but it is not difficult to get an intuitive grasp of the relationship between the volume of the shadow and the shape and orientation of the particle.

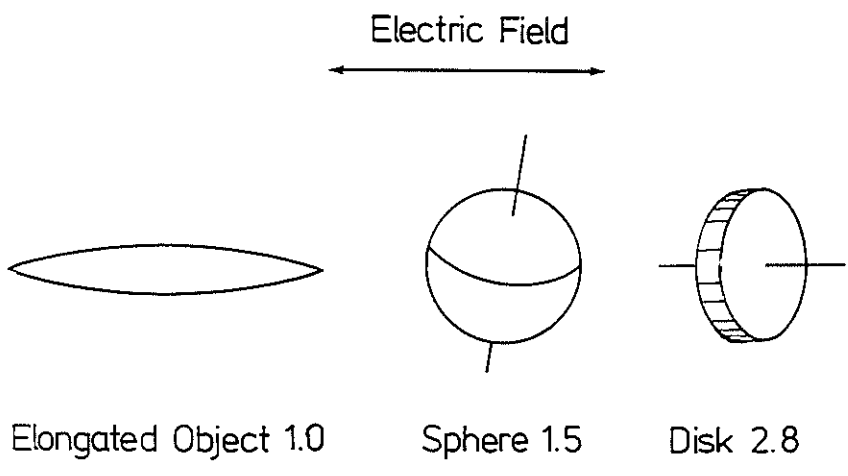

Fig 2. Shape factor for some geometric shapes with the same volume (49,63,67,70)

For instance, a disc oriented with its plane normal to the current field, will produce a shadow that is large in relation to its geometric volume (shape factor approx. 2.8). Position the disc with its plane in the axis of the current flow and the electrical volume becomes a better representation of the geometric volume. A long, slender body with its long axis moving parallel to the current field will have an electrical space volume only slightly greater than its geometric volume: its shape factor approaches 1.0. Thus the shape factor could be defined as the ratio of the signal pulse height produced by a given particle to the minimum pulse height that can be produced by a particle of the same volume (see Fig. 2).

3.4.3.3. Flow-induced (red) cell shape changes

Bagge et al (69) and Kachel (70,73) presented photographic evidence of the shape changes that rbc undergo when they are subjected to hydrodynamic stresses in apertures. They showed that normal rbc suspended in saline took on an ellipsoidal shape in fast liquid streams such as those found in Coulter apertures. This shape conforms very closely to the lines of current force that flow around it, causing the electrical shadow volume and the geometric volume of rbc to have nearly the same value. They found shape factors in the 1.06-1.12 range (mean 1.09). Similarly, they found a shape factor of approximately 1.5 for human lymphocytes and granulocytes as well as for fixed, shrunken rbc, lacking the ability to deform into a fusiform shape.

These facts can seriously affect the accuracy of cell volume (MCV) and indirectly

RDW, Hct and MCHC-determinations (37). Several detailed papers and reviews have been written on techniques of measuring rbc deformability of both normal and pathological subjects (39,74-79). Deformability is a basic characteristic of rbc; its regulating factors are presently well known: surface-volume ratio, internal viscosity and viscoelastic properties of the membrane (75,78,79). Herrera et al (75) have recently studied the deformability of 64 hereditary haemolytic anaemia rbc, using the Ektacytometer, a laser-diffraction viscometer (74). They found that deformability is always decreased (and consequently the shape factor is increased) in rbc membrane diseases and in haemoglobinopathies: on the other hand deformabilities were found normal in all the enzymopathies studied. The deformability decrease could be either due to one regulation factor (increase of internal viscosity easily demonstrated by ektacytometry in hereditary xerocytosis and haemoglobin CC) or to several parameters: increase of internal viscosity and decrease of surface-volume ratio in hereditary spherocytosis.

Bator et al. (39), using a novel method enabling monitoring of the deformability of individual cells, demonstrate that the measured MCV of 114.3 fl of a (homogeneous) sickle cell sample, actually would be 90.3 fl, if appropriate allowance would have been made of the shape factor of 1.38 instead of using a fixed factor of 1.09, since 114.3 x 1.09/1.38 = 90.3 fl.

Since, as we have seen, impedance-sizing flow hcy uses a constant (mean) rbc shape factor of 1.09 (39) for pathological or artificial rbc with decreased deformabilities (and consequently increased shape factors) the impedance signal over-estimates the rbc volume, that is the MCV and the Hct, and consequently underestimates the MCHC (37). The opposite

applies, albeit to a lesser degree, for rbc with increased deformabilities: the MCV and Hct will be underestimated and the MCHC will be overestimated (37). This was already mentioned in S 2.3.3. for polycythaemic patients with low MCH (p. 68).

The overall conclusion is that in impedance-sizing MCV and Hct will be found erroneously more extreme than the "true extreme value" at either extreme side of the mean, whereas the MCHC tends to be erroneously blunted, leading to erroneous constancy (cf S 2.3.3., p. 68).

3.4.3.4. Red blood cell sizing: MCV (Hct, MCHC) and RDW

3.4.3.4.1. MCV (Hct, MCHC)

All the main features pertaining to MCV (and consequently Hct and MCHC)-measurements have already been described, viz:

a. The mean of the micro-Hcts of several fresh, normal blood specimens as a reliable MCV calibrator for flow hcy instruments, provided 1.5% allowance for trapped plasma is made (S 2.3.2, p. 68).

b. MCV-errors due to abnormalities of the blood specimen such as hyperosmolar plasma, cold agglutinins, haemolysis etc.(Ch II, S 3.1.2.1).

c. MCV dependency on fluidic and electronic improvements (S 3.3.2, p. 75).

d. MCV dependency on flow-induced shape changes (deformability) (S 3.4.3.3, p. 87).

3.4.3.4.2. RDW

It will be evident that factors influencing MCV will generally also have their impact on the Red cell Distribution Width (RDW) and vice versa. As described in S 3.3.2., the fluidic and electronic techniques employed in

the different instruments and the shear stresses applied during the measurements can greatly affect the height and shape of the generated pulses and hence rbc size distributions. However, if artefacts can be avoided, these distributions, visualized in histograms, reflect the native non-discrete heterogeneity of rbc (80,83) and the RDW is an index of this natural variation in rbc size; it is a quantitative analogue of what is termed subjectively "anisocytosis", or heterogeneity of size, on the peripheral blood smear. Bessman et al (81) have recently described their important "Improved classification of anemias by MCV and RDW". Nutritional deficiency, whether iron, folate, or vitamin B12, always causes an increased RDW, the RDW being more sensitive than the MCV. In contrast, normal RDW accompanies pure hypoproliferative anaemias, resulting from chronic disease, marrow toxicity, or aplasia, independent of the MCV. Among the hereditary haemoglobinopathies there is a relation between anaemia and RDW: RDW is normal unless concomitant deficiencies are present (81,83). In increased rbc destruction from any cause and with any MCV, nonanaemic compensated disorders are homogeneous (normal RDW), whereas anaemic disorders are heterogeneous (increased RDW). Johnson et al (82) confirmed these findings in microcytosis for heterozygous thalassemia and chronic disease on the one hand (normal RDW) and iron deficiency on the other (increased RDW). Moreover, Bessman et al (81,83) show how rbc histograms identify rbc fragmentation or agglutination, dimorphic populations (e.g. by transfusions or iron therapy) and artifactual counting of lymphocytes as rbc.

An important feature of the RDW is the confusion brought about by the various prevailing RDW definitions and the ensuing potential pitfalls in RDW interpretation (84).

The RDW definition differs not only among the various Models of the Coulter Counter (53), it also differs among manufacturers. Whereas in the Coulter S-Plus-I the mean of the RDW values was equal to 10 (ten) by definition (53), thanks to the improved technology in the Model S-Plus-II (and subsequent Models) RDW could represent a final CV and is expressed as a percentage, the reference range being in the order of 13.3 +/- 1.5% (53,84). By a special computational technique the latter RDW is totally unaffected by artefacts (large plt, plt clumps or electrical interference on the left, and doublets, triplets, agglutinates, and aperture artefacts on the right) and consequently more precise and very sensitive to distribution variations (53,67). The Sysmex instrument CC-800 defines RDW as the "distribution width measured 10 percent of the distance from the base to the highest peak" (85), being only a direct measurement of the rbc distribution width, not divided by MCV to give a CV. With such a measurement when MCV is low, either a heterogeneous or homogeneous distribution will produce a normal RDW. When MCV is high, any rbc-distribution will yield a high RDW by this technique (84). Thus, use of a RDW-definition that is not a CV will not allow the important distinctions described by Bessman et al. (80,84). ICSH have issued proposed recommendations for the standardisation of cell size analyses to further comparability of results (86,87).

Although, perhaps, less suitable for routine clinical purposes, these RDW-problems can be avoided by using the actual rbc distribution histograms rather than their descriptive statistics. Moreover, these histograms can give a superior impression of the actual situation compared to figures alone. This is beautifully exemplified in several recent papers (81-83,88,89). Bessman et al

(81,83) and Scholda et al (89), using the Coulter Model S-Plus II, found valuable results in microcytic and macrocytic anaemias for diagnoses as well as for monitoring the therapy. They show that the mechanized volume distribution analysis is superior to the microscopic cell size estimation, except in spherocytosis (which, as we have described, is generally not appropriately sized by automated instruments due to deviating shape factors; S 3.4.3.3). Moreover, rbc histograms are quantifiable in contrast to blood smear analyses. This is the more important since individual people have their own rbc histograms (83), that can be reproduced very precisely (83).

3.4.3.5. Platelet sizing, MPV, PDW, Pct

Since the literature on plt sizing is proliferating, it is impossible to cover the subject in detail. Timely reviews and papers on the techniques, biological significance and clinical applications of plt sizing have recently been published (11,49,90-95). The major features will be briefly highlighted here:

a. Shape changes and MPV

Similar considerations about fluidic and electronic improvements (62,66) and about deformability and shape factor, as described for rbc (see above), apply to plt, although plt are much less deformable than rbc under shear stress (90,91). On the other hand, there is a substantial influence of the specimen temperature and of the type of anticoagulant on the MPV (94,98,99). EDTA, in contrast to citrate, causes plt to transform from an ellipsoidal shape to a sphere, resulting in EDTA/citrate-MPV ratios of 1.26-1.50 (90,94).

EDTA appears to cause a time dependent increase in MPV, probably due to 2 factors: shape change and, after a variable period of time, actual swelling due to water uptake (90,94-99). This phenomenon makes standardization in plt-analysis time and temperature imperative if reproducible and comparable MPV-values in EDTA-anticoagulated blood are to be expected (94-99) (MPV are constant after 2-4 hours). On the other hand, both plt count and MPV remained stable in blood collected in ACD/di-Na-EDTA anticoagulant for up to eight hours at room temperature; moreover this anticoagulant provided the best overall conditions of anticoagulation (98).

b. Platelet distribution and PDW

Plt volumes approximate a log-normal distribution (85,90,92,96), their heterogeneity being expressed as Platelet Distribution Width (PDW) (88). In order to avoid interference (e.g. due to microcytic rbc, electrical noise, megakaryocytes etc) in the Coulter S-Plus counters, stringent distribution criteria must be met for curves to be fitted and accepted (10,53). Different criteria are established in Sysmex instruments (85). Analogue to the RDW-situation, PDW definitions differ among the various Coulter S-Plus Models (53) and among different manufacturers: its reference value being 10 for Coulter's S-Plus I and 17-19 for Coulter's S-Plus II-V (53), whereas Sysmex defines PDW (identical to RDW) as the "distribution width measured 10 percent of the distance from the base to the highest peak (85).

c. Inverse, non-linear relationship between MPV and plt-count

In normal subjects there is an inverse, non-linear relationship between MPV and the plt count (88,94), necessitating the use of a nomogram for practical applications (Fig. 3) (94).

Characteristic departures from this relationship have been described for several diseases (88,94,100). Taking into account the nomogram, MPV is normal in hyperdestructive, is low in hypo-proliferative and is high in myeloproliferative diseases as well as in splenectomy (94,100). The inverse relationship implies that the "normal" Pct-value is also dependent on the plt count. Although the above

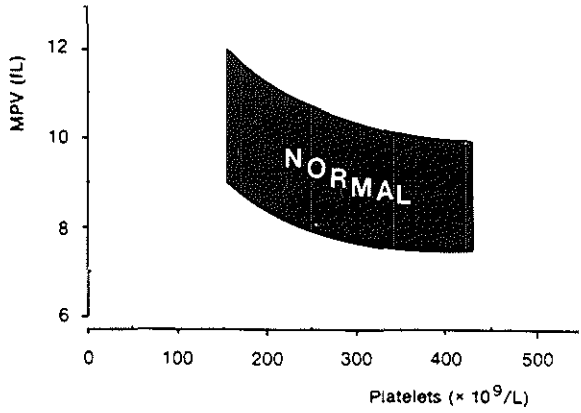

Fig 3. MPV-nomogram (Reprinted with permission of Coulter Electronics Ltd, Luton, UK)

shows that important clinical information can already be gained from MPV, the routine use of EDTA and the ensuing requirement for carefully controlled conditions impede the practical use of MPV in diagnoses (90). Problems are further compounded by the lack of comparison of MPV-values in different instruments due to both different MPV-definitions and different instrument responses to the available MPV QC-materials (101,102).

d. Platelet Rich Plasma (PRP)

If only older-generation routine instruments, not capable of simultaneous counting and sizing of rbc and plt, are available, plt will have to be separated from rbc. The most common method

used has been low-speed centrifugation, yielding so-called Platelet-Rich Plasma (PRP). However, centrifugation leads to variable plt counts and can lead to non-random exclusion of larger plt (90). This problem may be further aggravated in pathological conditions (90). Increasing g-forces progressively deplete PRP of its larger plt with a concomitant fall in MPV and PDW (103). Anticoagulants also affect these parameters (103). So strict standardization of PRP preparation is needed (103).

e. Overall conclusion

EDTA is less suitable for optimal routine determination of plt counts and MPV, because of time-dependent plt swelling and a relatively high incidence of aggregation (Ch II, S 3.1.2.1). The promising characteristics of low-pH citrate (or citrate/EDTA) should be further explored in order to reliably benefit from valuable MPV data that flow hcs could provide in daily routine (97,98).

3.4.3.6. White blood cell sizing: partial differentiation

Under the controlled conditions of lysis in the Coulter Models S-Plus II-V, partial differentiation of wbc by volume analysis has been accomplished: lymphocytes and granulocytes can be differentiated (10,49,67,88). In Coulter's Model S-Plus IV-D a third population, called monocytes, also includes blast and plasma cells, (post)myelocytes, promyelocytes and promonocytes, while granulocytes also encompass metamyelocytes, eosinophils and basophils (49,88). However, these histograms do not represent the actual volumes of wbc, in contrast to the rbc and plt histograms (10,49,88). The lysing reagent acts on the wbc membrane and cytoplasm, causing differential shrinkage of the wbc types, allowing the wbc to

be classified by their relative sizes (49,88). Richardson Jones (67) and Gibson (104) give a full account on the historical development of this method over the last two decades.

In several recent papers good correlation is described between lymphocyte counts given by the Coulter Models S-Plus II-IV and microscopic counts (105-108). Likewise, these Coulter Models correlated well with three other automatic wbc analyzers (Hemalog D, H 6000, Diff 3) (106). Consequently, applying this in daily practice, could substantially reduce the number of conventional differential counts by carefully prescreening by automatic wbc counting and sizing (105-108). However, it should be taken into account that high rejection rates and often considerable underestimation of the lymphocyte percentages were observed in neonates and infants as well as in infectious mononucleosis and in lymphoproliferative disorders.(108)

It will be obvious that adequate threshold setting is vital to the appropriate counting, sizing and (partial) differentiation of wbc. It should be remembered that these settings are dependent on the particular lytic agent, diluent and aperture tube used (8,11). Furthermore, it should be realized that QC materials invariably contain simulated rather than native wbc, being either fixed (human or avian) rbc, fixed wbc or latex particles (8). Thom (8) beautifully depicts threshold curves which manifest the discrepant behaviour to diluents and lysing agents between non-lysable, osmotically inert, wbc simulators and native wbc, which are liable to lytic and osmotic influences. Consequently, totally different pulses can be generated by originally equally-sized simulated and native wbc. In practice, adequate threshold setting is only possible with native wbc (8,11).

3.5 Optical (light-scattering) blood cell counting and sizing

Under carefully controlled experimental conditions light scatter signals from cells can give practical data on cell number and size. The signals can also contain much additional morphological information, but they are often difficult to interpret because the scattering process is a complex phenomenon. This process is a function of many cell characteristics such as size, shape, orientation, (relative) refractive index and properties of the nucleus and organelles, if any (109-117). For particles such as blood cells whose size is greater than the wavelength of the incident light, most of the scattered light is concentrated within a narrow angular region in the forward direction: the so-called Mie-scattering (109,113,114). The extent of this region is defined by the angle for which the intensity of scattered light reaches its first minimum. As the angle of observation increases beyond this minimum, the intensity of scattered light varies in an alternating sequence of maxima and minima produced by interferences of the reradiated waves from various portions of the cell (109). Due to the complexity of the scattering phenomena, practical results have come from empirically determined relationships between the scatter signals and cell characteristics (109-117).

The main representatives of light scattering instruments are Ortho's "ELT"-series (51,52,54,55) and Technicon's H-6000 and its predecessors (51,56-58) (cf S 3.3.2.1). In many respects their accuracy and precision are affected by factors common to all flow hcs irrespective of their detection system.

Special features of optical systems are:

a. A relatively small sensing volume that can be obtained by employing a hydrodynamically

focused sheathed sample stream and by focusing the incident light (cf S 3.3.2.1). For optical systems volumes as small as 2-3 pl are commonly obtained as contrasted with aperture-impedance sensing volumes that typically exceed 100 pl (113).

b. Although optical sensors have relatively small sensing volumes, it is generally necessary to apply a correction for coincidence, especially if the system is to be used to count both rbc and plt at the same dilution (113).

c. Cells are counted in laminar flow and hence undergo only a very small radial shear force that is zero at the center of the stream. Consequently, cells in laminar flow are free from longitudinal acceleration (37,113) in contrast to cells in aperture-impedance systems (S 3.4.3.3: flow-induced (red) cell shape changes). However, the typical mammalian biconcave-shaped rbc show (large) orientation-dependent variations in signal size (115,116). Both these differences as well as the dependency of optical sizing on cell refractive index can cause discordant MCV (Hct, MCHC) values of the same rbc in both systems (37-39, 54-56, 118,119).

d. Laser-illuminated systems have greater potential for resolution of the structural details of the cells than (incoherent) light-illuminated systems (113).

e. In Ortho's ELT-instruments discrimination between rbc and plt depends upon three cell variables : volume, refractive index (MCHC;37,120) and "time of flight" (114) through the sensing zone. Moreover, by using a dual detection system in the so-called "three-part" version, the light scattered in the forward direction can be used for counting the various cells, while the laser light collected at right angles to the incident beam can be used to probe the

cellular granularity of wbc. This enables a wbc population to be segmented into granulocytes, lymphocytes and monocytes (51, 52, 54, 55, 114, 121).

f. In Technicon's H-6000 and some of their older-generation instruments wbc differentiation is accomplished in a dual detection system where light scatter (depending on cell size) and light absorption due to cell peroxidase stain are simultaneously measured. In the XY-representation of scatter and absorption characteristics, movable thresholds divide the display into wbc subpopulations (51, 56-58, 113).

CHAPTER IV

PREPARATION AND EVALUATION OF INTERNAL QUALITY CONTROL MATERIALS FOR FLOW HAEMOCYTOMETRY

1. Prologue

As emphasized in Ch II, S 3.3, p.37 on HSLP in the analytical phase, control materials should play an essential role in the process of continuous self-auditing in daily hcy routine. The extreme cost of commercial controls generally is a serious impediment to their frequent use in daily practice, a basic requirement for optimal IQC (S 2.4). This important disadvantage was one of our main incentives to this study on QA in hcy in general and to the preparation of IQC materials in particular.

We describe the possible approaches to the preparation of IQC materials and conclude that for rbc QC, native rbc are required (S 2.3). In the course of our investigations reported below, we gradually gained insight in a basically different behaviour to haemolysis of electrical and optical hcy counters. As we describe in S 2.5 below, optical (light-scattering) instruments -- in contrast to electrical (aperture-impedance or resistive-particle) counters -- are sensitive to haemolysis, leading to decreasing rbc counts and increasing pseudo-plt counts. Since prevention of haemolysis appears to be a major problem in rbc preservation, preparation of IQC materials for electrical counters has been found much easier than that for optical instruments. The first five papers (S 2) deal with the descriptions of preparations and evaluations of IQC materials suited for electrical and to a lesser extent

for optical counters. The next two papers (S 3) are spin-offs of our ongoing research on the improvement of IQC materials for optical instruments. Since commercial 8-parameter IQC materials for the ELT-8 instrument do not yet exist (with one recent exception: Hyland's Lasercheck), a simple material for the instrument's plt counts is described (S 3.1). In the framework of our research on the further reduction of haemolysis, a sterile method for the age fractionation of rbc (S 3.2.) was developed. In S 3.3. a "comparison of the degree of haemolysis of young and old human red blood cells during storage" has been described. Although a highly significant difference between the degree of haemolysis of the fractions is found, the separation technique, unfortunately, does not yield a better starting material for IQC controls in optical instruments. Ongoing research on further reducing rbc haemolysis and pseudo-plt-formation is quite promising, but the procedures and results shall not be described here.

Finally, the rheological behaviour of patient and control bloods is shown to be comparable at shear rates probably occuring in hcy, provided their Hcts are taken into account (S 4).

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2.1.
Brief technical note

A white blood cell control of long-term stability

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Introduction

Adequate quality control (Q.C.) of white blood cell (WBC) channels of automated hematology instruments remains a major problem. Due to their extreme lability [1] and their great biological variability [2] native WBC are insuitable for most Q.C.-methods as recently reviewed by Koepke and Protextor [3]. Therefore, the only pragmatic approach is using (commercial) controls containing stable WBC-substitutes [4], preferably in conjunction with the use of a computerized 'average of normals' Q.C. program [5].

WBC-substitutes used in commercial controls consist either of latex particles or of fixed human or avian RBC [6]. However, these substitutes of potentially long-term stability are seldom offered separately, but rather as so-called multiparameter controls (perhaps for commercial or practical reasons). The stability of these controls generally does not exceed 2 months, due to the presence of (stabilized) RBC and/or platelets. Consequently, there is a continual need to employ new multiparameter controls, which almost invariably come from different lots of material. Hence, these may differ in composition and subsequently the continuity of control might be lost.

In this paper we describe the use of commercially available fixed human RBC as WBC-substitutes of long-term stability. To our knowledge, this is the first documented report on WBC quality control material exceeding 10 months' periods of stability.

Methods and results

Fixed human red blood cells (2.5 ml vials of 'Stabicells') can be bought from the Rijksinstituut voor de Volksgezondheid (RIV), Bilthoven, the Netherlands. A box of 6 vials (order No. 8011) costs about Dfl 95.00, equivalent to about $ 35.00. The red blood cells were fixed with formaldehyde by the (slightly modified) method of

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0009-8981/83/0000–0000.$03.00

Benedek [7] (Dr. P.W. Helleman, personal communication).

We did not attempt to make quantitative dilutions. After vortexing for 5 min, about 1 : 500 dilutions of Stabicells in Isoton or Isoton-Plus were made. For preservation 10% merthiolate was added in a final concentration of 1 : 2000. The suspension was divided into 30-ml glass injection bottles, that were kept at room temperature. Before use, the bottles were vigorously shaken by hand to suspend the cells then left undisturbed for at least 5 min to clear the foam and the air bubbles. (Persisting foam, if any, does not invalidate the results, provided the instrument's aspiration nozzle is kept below the foam.) Prior to aspiration the bottle was placed on a roller mixer or carefully inverted a few times to homogenize the suspension.

Measurements were originally made in a Coulter Counter Model S and more recently in a Model S-Plus II. The WBC channels of both models were monitored with the commercial controls Ortho Normal and Abnormal (Ortho Diagnostics, Beerse, Belgium). Moreover, Haem C control (Baker Diagnostics, Bethlehem, PA, USA) was used in the Model S and Hyland 8-parameter Hematology control (Hyland Diagnostics, Lessines, Belgium) in the Model S-Plus II. Accuracy was checked in a monthly regional Q.C.-survey and in the Ortho Quality Control Program. Recalibration was performed if judged necessary by the accuracy checks.

Table I shows the results of (1) one representative lot (Lot 1) of the four lots of suspensions measured on the Coulter Model S over the past 6 years; (2) the most recent suspension measured on a Model S-Plus II; and (3) the lymphocyte percentages as generated by the Model S-Plus II.

TABLE I

RESULTS OF THE WBC MEASUREMENTS IN THE COULTER S AND COULTER S-PLUS II AS WELL AS THE LYMPHOCYTE PERCENTAGES AS GENERATED BY THE COULTER S-PLUS II.

	n	$\bar{x}$ ($\times 10^9$/l)	SD	CV(%)	Lymphocyte (%)		
					n	$\bar{x}$	CV(%)
Coulter S							
Lot 1							
first 8 weeks	20	11.08	0.18	1.7			
total 19 months	99	10.98	0.31	2.8			
last 9 weeks	20	10.99	0.23	2.1			
Coulter S-Plus II							
Lot 2							
first 10 weeks	20	7.02	0.15	2.2	4	86.3	1.4
total 10 months	92	6.97	0.16	2.3	35	86.7	2.0
last 6 weeks	44	6.98	0.15	2.1	11	85.8	1.8

Discussion

Table I shows overall coefficients of variation (CV's) of 2.8 and 2.3% in the stated periods. These CV's compare favourably with an inter-assay CV of 3–6% for WBC as stated by Gilmer and Williams [4]. The differences in the means between the first weeks, the last weeks and the total periods are neither statistically ($p > 0.05$) nor clinically significant. Good linearity was shown by a 1 : 1 dilution of Lot No. 2 in Isoton-Plus giving a mean value of 3.45 ($n = 8$).

Initially quantitative dilutions were made to check the accuracy of the measurements. The discrepancies between the experimental results of lot 2 (mean value = 7.0) and the theoretical value of 7.6 (1.85 ml of Stabicells — concentration 4.12×10^{12} RBC/l – in 1.0 l of Isoton-Plus) can be explained by the suggestions of Thom [2]. He states that exact WBC counting, employing WBC-substitutes and using counters with a fixed threshold value calibration, is more or less a matter of luck, due to 'incompatibility' between simulated and native WBC. ('Incompatibility' meaning that patient blood and control blood cannot be measured with the same threshold level due to generation of different pulses in relation to frequency, shape and amplitude distribution [2].)

Discrepancies of results of WBC-substitutes can also be due to differences in measuring principles, notably the Coulter principle versus the laser principle. This is

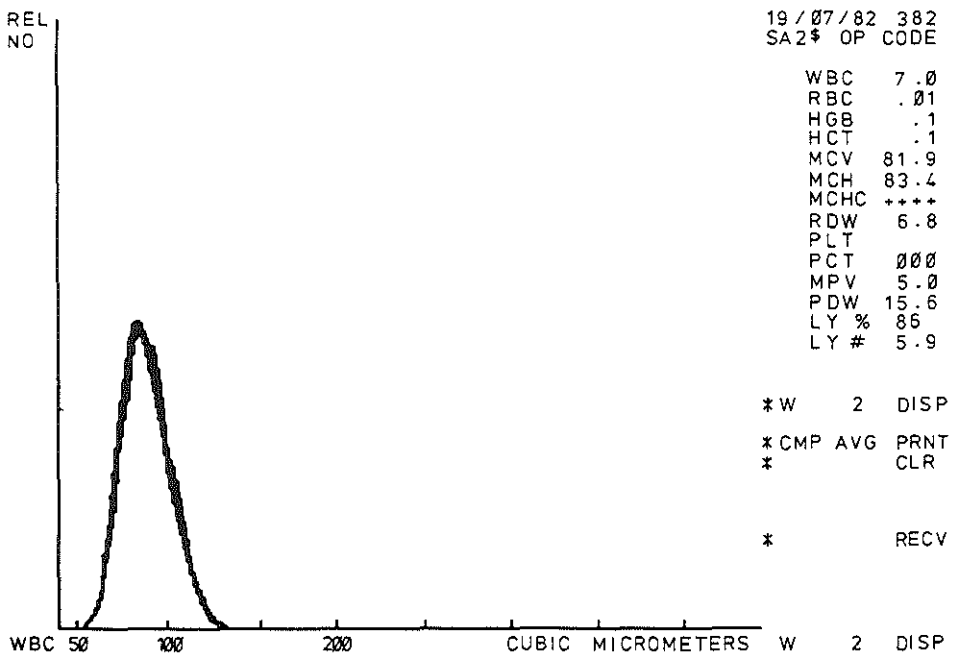

Fig. 1. WBC-Histogram of single aperture 2 (SA 2) showing total WBC and lymphocyte percentages display.

very pronounced with latex particles and fixed avian cells like chicken red blood cells (RBC). With Hyland's Normal 8-parameter Blood Control containing fixed avian cells, we found 6.6 ($n = 5$) in the Coulter Model S-Plus II against about 27 ($n = 5$; bad reproducibility) in the ELT-8 (Ortho Diagnostics). The histogram of these ellipsoid cells with round nuclei was found to be bimodal (not shown) due to their random orientation as they pass through the laser beam, as described by Loken et al [8] for forward angle light scattering. On the other hand we found 12.5 ($n = 2$) in the ELT-8 against 11.0 in the Coulter S for the fixed human RBC used in our Lot No. 1. Latex particles may also be used as WBC substitutes. However, apart from being very expensive, they bear the least resemblance to native cells, leading to very different responses in different instruments [9]. These examples clearly demonstrate that the values obtained from the two instruments, of the fixed human RBC (used as WBC substitutes) are in much closer agreement (12.5 and 11.0) than those of avian RBC (27 and 6.6) and latex particles [9].

There is no commercially available reference control material suitable for monitoring the performance of the lymphocyte percentage (ly %) function in the Model S-Plus II [10]. Therefore, this function should be evaluated at least daily by comparing the conventional differential results for all samples with the results reported by the Model S-Plus II [10]. Generally, after analysis of our suspension a ly % print-out is not automatically obtained, but, the percentages of the individual channels can be printed out instead (Fig. 1). Although the suspension cannot be used to check on the proper functioning of the shrinking process of lymphocytes, it can be used to monitor the proper ly % calculation (Table I).

In conclusion, we show that Stabicells are a simple, inexpensive Q.C. material for monitoring long-term WBC reproducibility. They are, however, not suitable for absolute WBC calibration.

Acknowledgements

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2.2.
A stable human platelet-white blood cell control for the Coulter Model S-Plus II

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Summary

The preparation and evaluation of a human platelet-white blood cell control of at least 5 months' stability, as measured in the Coulter Counter Model S-Plus II, is described. In some preparations minor departures from log normal platelet (PLT) distributions were found, impeding the generation of values for mean PLT volume (MPV) and PLT distribution width (PDW). In many cases, however, excellent linearity for PLT and white blood cell (WBC) counts and low coefficients of variation for PLT and WBC counts and PDW were found. Some possible factors causing the large coefficient of variation found for MPV values are mentioned.

Introduction

Once the calibration of platelet (PLT) channels of the Coulter S-Plus II has been performed [1–6], stable (and preferably inexpensive) materials are needed to monitor the instrument's performance and drift [7]. Human PLT-counts in normal EDTA-anticoagulated blood are stable for a few days, provided the blood is intermittently mixed [8]. As a consequence of this short-term stability, EDTA-blood is not suitable as a control.

PLT-control samples have always proven to be most difficult to manufacture [9]. Hence PLT-substitutes are employed such as human and animal PLT (stabilized or fixed), animal red blood cells and plastic particles [1,3–7,10]. However, there are problems encountered with these materials [1–7,10]. These include: (1) clumping of PLT [1,2]; (2) non-matching of PLT substitute distribution with the actual human PLT distribution [3–5]; (3) cumbersome fixing procedures [6]; (4) discrepancies between the results obtained from two types of instruments: resistive-particle

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(Coulter-type) instruments and laser-based instruments. These are brought about by differences in measuring principles [10].

The above suggests that human PLT are very labile. Nevertheless, in this paper we describe a simple method for the preparation of a stable, unfixed human PLT-control used in a Coulter Model S-Plus II. Furthermore, we show that this PLT-control exerts no adverse reactions with the stable white blood cell (WBC) control we recently described [11], thus providing a human PLT-WBC control of at least 5 months' stability.

Materials and methods

Preparation of a PLT-concentrate from buffy-coats

Human PLT-concentrates are made from fresh buffy-coat layers within 24 h and kept at room temperature to preserve the normal, discoid PLT shape [12]. All preparations are made under sterile conditions. Four buffy-coats, each about 70 ml, left over from the routine processing of red blood cell concentrates, are pooled into an empty, 600-ml transfusion bag. Acid citrate dextrose (ACD; NIH formule A) is added until the bag is full. The bag is centrifuged at room temperature for 15 min at $400 \times g$. After centrifugation, the bag is carefully placed in a plasma extractor that is routinely used in any blood bank (Fenwal, Travenol Laboratories [13]). The platelet rich plasma (PRP) is carefully transferred into a double transfusion bag via a transfer tube until a 2-cm PRP layer is left with the WBC-precipitate. The PLT are concentrated by recentrifuging the PRP in a double bag at room temperature for 5 min at about $2000 \times g$. The centrifuged bag is placed in a Fenwal plasma extractor and the supernatant (ACD) is transferred into the satellite bag until some 50 g of PLT-precipitate with ACD are left over in the primary bag. After sealing, the transfer tube is broken and the satellite bag is discarded. The PLT-precipitate is easily resuspended in the ACD solution by agitation. The suspension should be very fine at this stage (indeed no aggregates should be seen at microscopic inspection). The suspension is kept at room temperature before processing, which preferably should be done within 24 h [12].

The preservation medium

The preservation medium consists of ACD plus the following substances: albumin and gentamicin in final concentrations of about 2.5% and 400 μg/ml or more, respectively. Distilled water is added until the osmolality is about 300 mOsm/kg. Since it is one of our goals to combine this PLT-control with a WBC-control, Stabicells acting as WBC-substitutes (in a final dilution of about 1 : 500) are added as well [11]. This preservation medium can be made beforehand in a separate transfusion bag. Appropriate dilutions can be made by connecting the two bags via their injection ports using Fenwal site couplers and a transfer tube [13]. A 1 : 4 dilution of the PLT-concentrate described above will result in approximately 200×10^9 PLT/l, indicating a high yield (as we produced the PLT-concentrate from four buffy-coats).

Instruction for use

During storage the control is refrigerated in 6-ml glass bottles. Before use the bottle is shaken to resuspend the PLT and left for at least 10 min (allowing the suspension to come to room temperature and to clear it of air bubbles and foam). When needed, the suspension is carefully inverted to ensure homogeneity. Residual foam, if any, does not invalidate the results, provided the suspension is aspirated from below the foam layer.

Quality control of the Coulter S-Plus II

The Coulter S-Plus II was routinely monitored with three levels of Hyland 8-parameter control. (Travenol labs, Deerfield, IL 60015, USA). Accuracy was checked once a month in a regional quality control survey. Recalibration was performed if judged necessary by the accuracy checks.

Results

Several controls were prepared and were found to give reproducible PLT, platelet distribution width (PDW) and WBC results. Table I shows the results of the PLT-count, the mean platelet volume (MPV) and the PDW of a representative control during a 5-months period. Since the WBC results are comparable to those previously described [11], they are not listed. Our preparation gave similar CV's for PLT and PDW values to those of the Hyland Normal Control. This is displayed in Table I. However, the CV of the MPV in our control is very inferior to that of Hyland.

Table II shows non-significant differences ($p > 0.10$) between several measuring periods of PLT-counts and PDW indicating their perfect stability during a 5-months

TABLE I

THE PLT-NUMBER, MEAN PLATELET VOLUME (MPV) AND PLATELET DISTRIBUTION WIDTH (PDW) MEASUREMENTS OF THE HYLAND NORMAL 8-PARAMETER CONTROL AND OUR CONTROL IN THE COULTER S-PLUS II

The total measuring period is 5 months.

	PLT-count		MPV		PDW	
	$\bar{x}$ *	CV **	$\bar{x}$	CV	$\bar{x}$	CV
Hyland Normal Control, Lot 823 H_2						
Stated value:	225	5.1%	8.1	2.5%	not stated	
($n = 66$)	228.1	3.8%	8.4	2.7%	16.7	2.4%
Our control ($n = 66$)	168.2	3.8%	9.1	12.8%	17.3	2.3%

* Mean value.

** Coefficient of variation.

TABLE II

THE MEANS OF THE FIRST 10 MEASUREMENTS, THOSE OF ALL 66 MEASUREMENTS AND THOSE OF THE LAST 4 MEASUREMENTS FOR PLT-COUNTS AND PLATELET DISTRIBUTION WIDTH (PDW)

The non-significant differences between them indicate perfect stability of PLT and PDW over the 5-months period.

	n	PLT-counts		PDW	
		$\bar{x}$ *	SD **	$\bar{x}$	SD
First 12 days	10	168.4	4.7	17.6	0.3
Total period of 5 months	66	168.2	6.4	17.3	0.4
Last day	4	172.0	5.0	17.3	0.2

* Mean value.

** Standard deviation.

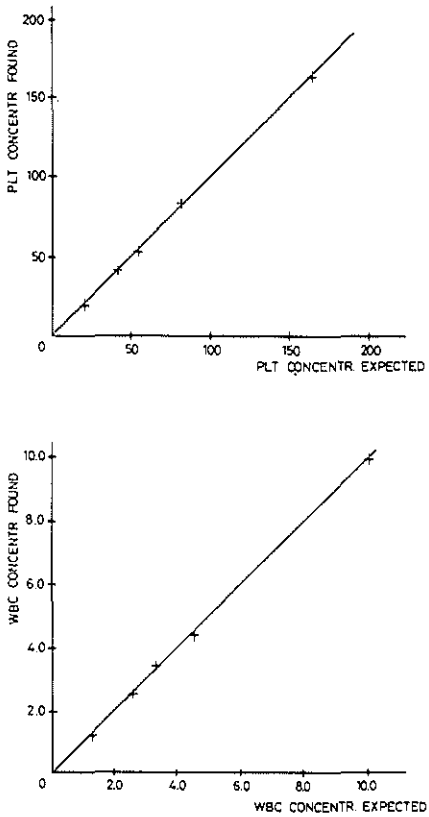

Fig. 1. Dilution of the 8-week-old control in Isoton Plus shows excellent linearity for both PLT and WBC counts.

TABLE III

MEASUREMENT DURING 5 CONSECUTIVE DAYS OF THE 8-WEEK-OLD PLT SUSPENSION

After 8 weeks in the refrigerator the PLT-suspension was brought to room temperature for this 5-day measuring period.

	n	$\bar{x}$ *	SD **	CV(%) ***
PLT	5	163.4	4.0	2.4
MPV	5	7.96	0.3	3.8
PDW	5	17.48	0.16	0.9

* Mean value.
** Standard deviation.
*** Coefficient of variation.

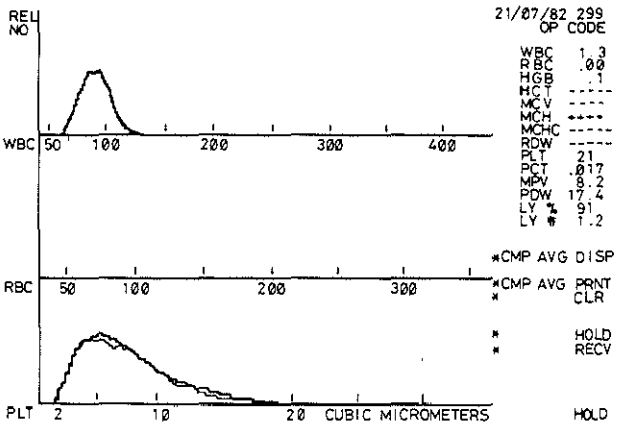

Fig. 2. Histogram of the 1 : 7 dilution of the 8-week-old control in Isoton Plus.

period. However, these comparisons are not meaningful for MPV due to the very poor CV found for MPV in our control.

Measurements were made for 5 days after the 8-week-old refrigerated PLT-suspension was brought to room temperature. Table III displays the results suggesting that storing the suspension at room temperature is also possible.

Fig. 1 shows excellent linearity of the 8-week-old control of both PLT and WBC counts with the dilution.

Fig. 2 reproduces the histogram of the 1 : 7 dilution of the 8-week-old control with Isoton Plus.

Discussion

There are several arguments for the preparation and description of a home-made PLT-control:

(1) the high costs of commercial quality control (Q.C.) materials inhibit their frequent use necessary to assure optimal intralaboratory Q.C.;

(2) a thorough search of the literature reveals no simple methods of preparing a PLT-control [1,4–7];

(3) patent literature is less accessible. Our enquiries with some commercial firms revealed that, for respectable reasons, they were not prepared to disclose to us any patent numbers or details relevant to their Q.C. materials. Moreover, a search in the Dutch Patent Office revealed that patent literature is lagging behind [14,15]. Furthermore, the invention described may well only be part of a procedure, and following it gives no guarantee of a successful product. Last but not least, the information is patented and consequently not publicly applicable;

(4) the (sole) use of commercial Q.C. materials contributes to the ever growing dependency of medical laboratories on commercial firms;

(5) sophisticated home-made intralaboratory Q.C. materials might be used together with commercial controls and Bull's algorithm on patients' data [16] to optimize intralaboratory Q.C.;

(6) due to the ever growing demand for transfusions with pure red blood cell concentrates, buffy-coats often have to be separated from red blood cells. Although there are several useful applications for buffy-coats (e.g. therapies with fresh PLT concentrates; interferon preparations), in blood bank practice they are sometimes left over and discarded.

Some of these buffy-coats, harvested from blood anti-coagulated with citrate phosphate dextrose-adenine (CPD-A) (pH about 7.2 at 20°C), were used as starting materials in our preparations. However, the PLT were diluted with acid citrate dextrose (ACD) (pH about 5.5 at 20°C), a crucial material for the successful preparation of the PLT-control. (It has long been known that acidification of PLT-concentrates results in aggregate-free suspensions [17]). Similarly, fine PLT-suspensions (as viewed under the microscope) were obtained, whereas PLT-clumps were prevalent in the more alkaline CPD-A. Indeed, when the CPD-A suspensions were employed for the PLT-controls non-reproducible results were obtained (not shown).

Albumin could also be an important ingredient in the preservation medium as it is known to provide the PLT with a 'protective coat' which diminishes the loss of PLT-constituents into the surrounding medium [18,19]. Moreover, albumin has a complex influence on the glass-adherence of the PLT [19]. In fact, we believe that albumin should be added to the PLT before bringing them in contact with any glass.

The results show an excellent CV for PLT-counts indicating that our WBC-substitutes of long-term stability [11] do not exert any adverse effects on the human PLT, notably no adherence of the PLT to the WBC-substitutes. This enabled us to prepare a human PLT-WBC control of at least 5 months stability.

The results also give an excellent CV for PDW. The CV for MPV, however, is very unsatisfactory. PLT swelling possibly is the main reason for this phenomenon [20]. The primary cause of such swelling is possibly the inability of the cell to maintain its volume in response to the continuing influx of sodium and water which results from the colloid osmotic effect of cellular macromolecules [20]. In a separate experiment (not shown) we attempted to prevent this swelling by adding mannitol

(2% final concentration) to the preservation medium. As mannitol is known not to penetrate PLT, its presence could counterbalance the colloid osmotic pressure of the PLT. However, this still did not result in MPV stability.

Many other factors, such as the storage temperature, the type of anticoagulant, the pH and the resulting PLT-shape changes appear to affect the (apparent) MPV [20–23]. However, the handling of this most complicated matter is beyond the scope of this paper and would require further extensive investigation.

Unfortunately, about one-third of our PLT-preparations did not show a perfect log normal distribution, giving rise to error code 10. Further studies will be necessary to clarify this phenomenon. We observed this same departure from log normality with the most recent Coulter platelet controls CTC-4 (Lots 32A and 32B), despite strict adherence to the instructions for use. The same disadvantage applies to the recently described PLT-control material PlasCon [7].

The availability of this control offers the possibility of implementing the internal quality assurance protocol advocated by Gilmer and Williams [24]. Once the calibration of the instrument has been performed according to one of the approaches described in this paper and/or those in the literature [1–6,24], an inexpensive, independent source of stable cell control is available in sufficient amounts to check the 'primary calibration material' for any deterioration or vial variances, to frequently monitor instrument drift and other gradual malfunctions and to promptly trace any instant instrument failures.

In conclusion, we propose a solution to some of the problems of PLT-manufacture mentioned in the introduction. We have described a simple preparation of a human PLT-WBC control with at least 5 months stability, suitable for the Coulter S-Plus II.

Acknowledgements

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2.3.

Preparation and evaluation of a 7-parameter intralaboratory control blood of 4-month stability

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SUMMARY Sterile, fresh concentrates of human red blood cells suspended in a specially designed sterile preservation medium have a stability of all six red blood cell parameters of over four months as measured in the Coulter Model S-Plus II. Substitution under sterile conditions of the unstable white blood cells for (commercially available) fixed (human) red blood cells is a well-established method to obtain simulated white blood cell suspensions of long-term stability.

In flow haemocytometry there are two different approaches to intralaboratory quality control (QC*): either the analyses of patients' data or those of control samples may be used.[1] As these approaches have their advantages and disadvantages, a combination of both is advantageous.[1–3] Use of patients' data requires computer facilities to calculate the patients' mean parameters, moving averages, moving modes, etc.[1–4] In this paper we will concentrate on the preparation and evaluation of a human blood control.

As the haematological parameters in EDTA-blood are stable for only a few days[5] artificial QC materials have to be used. A thorough search of the literature shows an amazing paucity of data on the characteristics[6,7] let alone on the preparation of QC materials.[1,2,8] Data on the preparation of QC materials may perhaps be found in the less accessible patent literature.[9,10] The (sole) use of commercial QC materials contributes to the ever growing dependency of medical laboratories on commercial firms. Moreover, the high cost of commercial controls inhibits their frequent use to assure optimal QC.

In this paper we describe and discuss a simple blood processing procedure to prepare a blood QC material and evaluate the results as measured in a Coulter counter Model S-Plus II.

* *Abbreviations* wbc white blood cells; rbc red blood cells; WBC White Blood Cell counts; RBC Red Blood Cell counts; Hb Haemoglobin; MCV Mean Cell Volume; MCH Mean Cell Haemoglobin; MCHC Mean Cell Haemoglobin Concentration; RDW Red cell Distribution Width; Mhct(s) Microhaematocrit(s); Chct Coulter haematocrit; QC Quality Control.

Materials and methods

MEASUREMENTS

Mhcts were determined after spinning the blood in capillaries for 5 minutes at about 12 000 *g* in a Hettich Mikro Rapid centrifuge. In the supernatants of spun blood, spectrophotometric ultramicro-determinations of Hb (as cyanmethaemoglobin) were performed according to Rice.[11] Blood pH was measured in a Radiometer ABL-2 instrument and osmolality in a Knauer osmometer. Methaemoglobin was determined according to Fairbanks.[12]

CALIBRATION AND QC OF THE COULTER S-PLUS II

Hb was calibrated with the international standard.[13] The other parameters were calibrated against the means of the Coulter counter values in the Ortho QC programme (Ortho Diagnostics, Beerse, Belgium). These means were shown to be valid by the finding that the means of 100 normal outpatient values[2] closely corresponded to the means of the laboratories' reference values of these parameters. The Coulter counter was monitored with Hyland 8-parameter control (Travenol Laboratories.[14])

BLOOD PROCESSING

Blood was collected in 75 ml acid citrate dextrose (ACD)-anticoagulant in a regular double plastic bag system. After centrifugation for 5 minutes at 5000 g the plasma and buffy coat were carefully pressed out in a Fenwal bag extractor.[14]

Remaining wbc and platelets were then removed by filtration through cotton wool in a closed sterile

system. We used the filter column from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (POB 9190), Amsterdam. This routine technique has been shown[15] to be the method of choice for the preparation of wbc-free and platelet-poor rbc concentrates. After centrifuging and removal of the saline, the sterile preservation medium (see below) was added through a port in the bag using a standard connecting tube and site coupler.[13] The final pH was 7·0. Under occasional mixing, aliquots of about 5 ml of the 'wbc'- rbc-suspension were aseptically filled through the connecting tube into sterile 6-ml screw-capped glass bottles in a laminar flow cabinet. Blood cultures were found to be negative in a randomly chosen bottle.

THE PRESERVATION MEDIUM

All chemicals used were of analytical grade. The next preservation medium was prepared and sterilised at 120 C for 30 minutes: 5 mM Na_2HPO_4, 30 mM K_2HPO_4, 15 mM K_2-EDTA. $2H_2O$, mannitol 2·2%, gentamicin 1 g/l, 4N NaOH until pH 8·5. After cooling, sterile albumin and glucose solutions were added under sterile conditions in a final concentration of about 3% and 0·5%, respectively. After vortexing for a few minutes about 1 ml/l of Stabicells (sterile, aldehyde-fixed rbc) were aseptically added to simulate wbc.[16] The final osmolality was measured (about 315 mOsm/kg) and set at approximately 300 by the addition of sterile distilled water.

Results

Figure 1 depicts the excellent stability of WBC, RBC, and rbc primary parameters and Mhct during a four-month period, automatically resulting in stability of the calculated parameters Chct (RBC · MCV), MCH (Hb/RBC), and MCHC (Hb/Chct). After this time the MCV gradually increases. WBC,[16] RBC, and Hb values are unchanged even after eight months. All the parameters remained constant after mixing the 4-month-old blood on a roller mixer for 6 hours, showing good mechanical resistance and stability of the control.

Figure 2 shows the supernatant Hb-concentrations in Mhct-tubes after centrifugation of a representative, normal EDTA-blood (kept at 4 C) (curve A) and of the described control (curve C) at different stages of the test period. Curve B represents supernatant Hb-concentrations as depicted by Spaethe.[6] A yellow supernatant colour corresponded to a Hb-concentration of about 0·3 mM, a yellow-brown colour to approximately 0·6 mM Hb, a brown-red colour to about 1·3 mM Hb. Higher concentrations of Hb showed red supernatants with progressively less sharp interfaces with the packed rbc due to increasing haemolysis. We consider supernatant Hb-concentration a good and practical criterion for rbc-stability, as the degree of haemolysis reflects the ability of rbc-membranes to withstand the trauma of high centrifugal forces. Figure 2 unambiguously

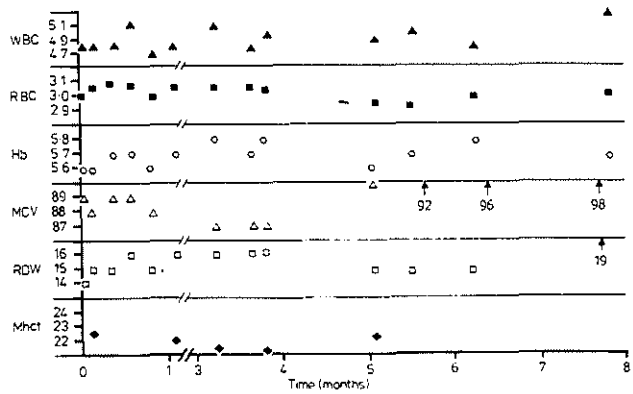

Fig. 1 *Stability testing of the described 7-parameter control blood during an 8-month period. All primary parameters are shown to be constant for four months, automatically resulting in stability of the calculated parameters Chct (RBC · MCV), MCH (Hb/RBC), and MCHC (Hb/Chct). Mhct determinations are also stable during the test period. WBC, RBC, and Hb are even stable during storage for eight months. (The units on the y-axis are: WBC in 10^9/l; RBC in 10^{12}/l; Hb in mM; MCV and RDW in fL, and Mhct in %).*

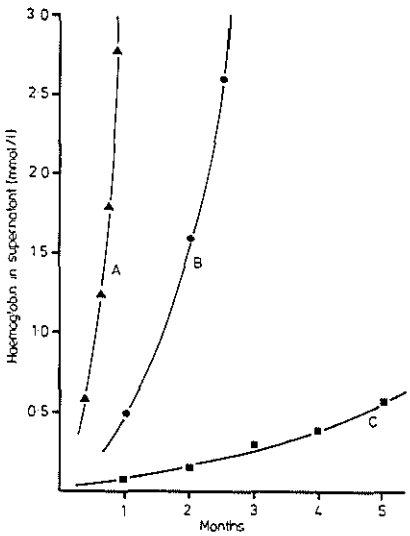

Fig. 2 *Supernatant Hb-concentrations in Mhct-tubes after centrifugation of a representative normal EDTA-blood (kept at 4°C) (curve A) and of the described control (curve C) at different stages of the test period. Curve B represents supernatant Hb-concentrations as described by Spaethe.[8] The figure shows the superior resistance to haemolysis of the described control.*

shows the superior mechanical resistance of the described control.

Due to the gradual loss of 2,3-DPG, which occurs during storage of blood,[17] the oxygen affinity of our control was markedly increased,[17] leading to a light red colour of the blood during the whole four-month period. However, methaemoglobin was gradually formed and amounted to about 7% after four months in our control. After this period the colour of the control gradually darkened due to increasing methaemoglobin formation.

Discussion

Commercial interests and the many problems encountered in blood cell preservation[1 7 8] are probably the reason for the surprising scarcity of literature on the subject.

PROBLEMS PERTAINING TO THE PRESERVATION OF BLOOD CELLS

The main problems pertaining to the preservation of blood cells are:

1 wbc and platelets decrease in a few days[9] and cause microaggregates.[18] Formation of microaggregates can be avoided by removing wbc and platelets by filtration, as described in this paper.[15]

2 It is common practice to store transfusion blood at 4 C. However, at this temperature the Na-K-ATP-ase pump is insufficiently active to maintain the normal cation-gradients in the rbc.[17] Consequently, K-ions are expelled and Na-ions and water are taken up, resulting in rbc-swelling and thus a MCV (and haematocrit) increase.[17]

3 Storage at 20 C or 37 C results in even quicker swelling of rbc[7] accompanied by a rapid darkening of the colour due to methaemoglobin formation (own experiments, not shown).

4 Accumulation of lactate due to glycolysis, resulting in a pH decrease. Lactate leaves rbc only slowly through a carrier-mediated process[19] and therefore exerts an osmotic effect.

5 Gradually complete loss of ATP and 2,3-DPG.[17]

6 Substitution of chloride ions for 2,3-DPG, also causing an osmotic effect since chloride exerts about 3·7 times the osmotic effect of 2,3-DPG.[19]

7 ATP-depletion leads to discocyte-echinocyte transformation, and ultimately echinocyte blebs start to bud off as spectrin-free vesicles.[20 21]

APPROACHES TO THE PREPARATION OF QC MATERIALS

At first sight several materials might be suitable to check the measurements of rbc and their parameters.

1 Artificial particles

None of the artificial spheres tried in the past, such as pollens, mould spores, yeast, polysterene latex or other plastic polymers, have proved suitable.[7] Moreover, it is necessary for several reasons that rbc in multiparameter quality control materials can be *lysed*. In blood flow cytometers the enormous mass of unlysable rbc-substitutes of approximately the same size as wbc would make measurement of wbc impossible. Hb determination of an added haemolysate would be seriously biased by light scattering. Furthermore, there would not be a check on the proper functioning of the lysing process. The very high cost of the artificial particles needed in such high quantities if they are to replace rbc is also disadvantageous. Finally, there might be a danger of clumping and possibly clogging of the apertures. This implies that the use of rbc is practically unavoidable.

2 Aldehyde-fixed or glycerolised rbc

There are a number of disadvantages to the use of aldehyde-fixed rbc[1 7] such as a tendency to clump and to adhere to walls of containers, rbc-rigidity resulting in erroneously high Mhcts, and the danger of incomplete haemolysis during the very short lysing

period used in modern blood flow cytometers.[22] These facts may lead to loss of accuracy and precision. In fact undiluted fixed cells are not suitable for use in fully automated counters.[1] Low temperature preservation of rbc in glycerol leads to progressive lysis[7] and an unphysiologically high and inconstant MCV (own experiments, not shown).

3 Outdated blood

The use of outdated bank blood has been advocated in the literature.[7 23] Washed, outdated rbc suspended in Isoton[®] and substituted with aldehyde-fixed rbc to simulate wbc[16] were successfully employed in the first author's laboratory for many years, allowing for the use of unlimited volumes of inexpensive daily QC material (unpublished). There are, however, some disadvantages to the use of outdated blood. Firstly, expired blood is more labile to the high centrifugal forces during a Mhct determination, causing considerable haemolysis and decreasing Mhct values. Secondly, after some three weeks the MCV of outdated rbc becomes progressively more sensitive to temperature influences and to mechanical stress during prolonged stay on a roller mixer. Thirdly, in modern so-called 8-parameter flow cytometers (which measure platelets simultaneously with wbc and rbc parameters) outdated blood is less suitable as a QC material due to interferences of rbc fragments in the platelet range. Studies on the addition of human platelets to the described 7-parameter (fresh) blood control are currently being performed in our laboratories.

Because all the materials described above have more or less serious disadvantages, we studied the possibility of preserving fresh rbc without fixation.

THE PRESERVATION MEDIUM

In this study we report the results of experiments in which monovalent cation exchanges due to gradients and concomitant MCV changes are avoided by suspending the freshly drawn rbc in a high potassium, low sodium medium of approximately the same concentrations as potassium and sodium concentrations inside the rbc. Preliminary tests showed that an impermeable osmotic substance in a fairly high concentration was necessary to counterbalance the impermeable Hb and other osmotically active substances such as lactate. Several substances are known to be impermeable to rbc (sucrose, mannitol, citrate[21 25], etc). We chose mannitol because of its neutrality.

Figure 3 shows the oncotic effect of mannitol and the relationship between the final mannitol percentage and the change of MCV; it can be concluded that a concentration of about 2·2% mannitol will lead to a constant MCV during at least 25 days.

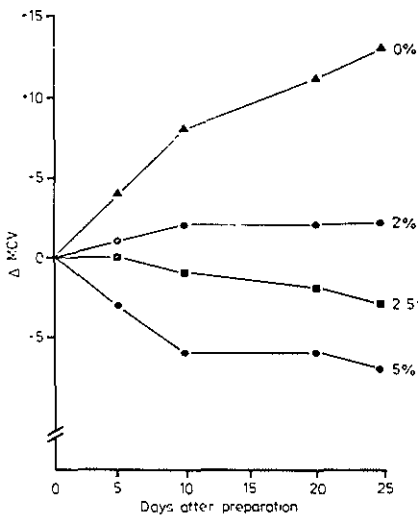

Fig. 3 Oncotic effect of mannitol. Example of MCV changes as a function of storage time at 4°C and the final mannitol percentage in the preservation medium. The conclusion can be drawn that a concentration of about 2·2% mannitol will lead to a constant MCV during at least 25 days.

Three per cent albumin was added for two reasons: firstly, to provide rbc with a protective coating;[26] secondly, to simulate the viscosity of fresh blood. It is generally accepted that the ideal control should mimic the patient sample as closely as possible.[7]

EDTA was added in a final concentration of 15 mM because this concentration is said to inhibit 75% of vesicle-formation.[20] However, there is a disadvantage in the use of such high concentrations of EDTA. It has long been known[27] that high concentrations of EDTA cause shrinkage of rbc in proportion to the excessive concentration of the anticoagulant. It is of practical interest that this phenomenon leads to erroneously low Mhcts but does not produce an error in Chct. The Chct is the correct value because the high dilution of blood in isotonic medium restores the shrunken red cells to their initial size.[27] This discrepancy prevents the Mhct being used as a primary method of calibration. We found a Mhct of about 22% against a Chct of 26%. Subsequent studies (not shown) strongly suggest that high EDTA concentrations are not necessary to yield stable control values in the Coulter S-Plus II.

CONSTANT HB- AND RBC-VALUES DESPITE GRADUAL OXIDATION AND HAEMOLYSIS

In time gradual oxidation of Hb eventually leads to dark-coloured blood due to increasing met-Hb formation.[28] However, Hb values remain constant for at least eight months as both Hb and the met-Hbs are converted to cyanmet-Hb.[13 28] Likewise, haemolysis gradually increases both spontaneously and after high-speed centrifugation. Analogous to Gear's[29] observations in a Celloscope counter, we found that under the geometric and electric current circumstances in the measuring apertures of the Coulter S-Plus II, rbc ghosts (lysed rbc) give MCV-values considerably smaller than those of intact rbc. We found (not shown) MCV-values in the 50–70 fl range in water-induced rbc-haemolysates. However, addition of (solid) sodium chloride (9 mg/ml) resulted in a MCV-shift to the right of about 12 fl, confirming that MCV is also dependent on the salt concentration of the suspending medium.[29] As 'pulses from the rbc-bath representing cells 36 μm^3 and greater are classified as red cells,'[30] haemolysis does *not* affect RBC. This explains why RBC are constant for eight months and probably much longer.

The above results show that the proposed high potassium, low sodium medium in combination with 2·2% mannitol for osmotic balance is a suitable preservation medium for freshly drawn rbc, particularly for maintaining a constant MCV. To the best of our knowledge this has never been described before. Thus fresh rbc provide a suitable 7-parameter control blood in combination with fixed rbc, which had already been shown to be stable wbc-substitutes.[16]

CALIBRATION AND QC

The availability of the described control makes it possible to implement the internal quality assurance protocol advocated by Gilmer.[31] Once the calibration of the instrument has been performed according to the method used in this paper or to one of the procedures described by Gilmer,[31] an inexpensive, *independent* source of cell control of medium-term stability is available to check the 'primary calibration material' for any deterioration or vial variances. After removal of the plasma proteins from different blood units, rbc with different antigenic make-up can be mixed with no apparent damage to the cell membranes.[6] This provides quantities of control blood large enough to minimise the frequency of handover from old batches to new ones, to monitor *frequently* drift and other gradual malfunctions, and to trace promptly any abrupt instrument failures.

We thank Dr JA van der Does, director, and Mr JK Jagdewsing, chief technician, of the regional blood-bank 's-Gravenhage en omstreken' for their hospitality. Without their help and advice the experiments presented here could not have been accomplished. The skilful technical assistance of Ms Anita Robers and Mrs Angela in 't Veld is also gratefully acknowledged.

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2.4.
Laboratory preparation and evaluation of a multiparameter hemocytometry control

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Key words: Hemocytometry; Internal quality control; Medical audit; Multiparameter control; Platelet fixation

Summary

A protocol for the laboratory preparation of a multiparameter hemocytometry control is given. Human platelets, stabilized by a basically simplified and inexpensive fixation procedure, are added to our previously described white and red blood cell control. Evaluation of this multiparameter control shows good precision characteristics and acceptable mechanical stability for at least 7 weeks, as measured in the Coulter counter Model S Plus-II. The control can basically contribute to the realization of the essence of internal quality control: continuous self-auditing and continuous attempts at improvement of performance.

Introduction

Perusal of the recent literature has shown that protocols for the laboratory preparation of so-called multiparameter quality control (QC) materials, including platelets (plt), for flow hemocytometry have never been published [1–5]. In a recent

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Abbreviations (and dimensions if applicable): ACD = acid citrate dextrose; AP = aperture; AVG = average; CPD-A = citrate phosphate dextrose-adenine; CSP-II = Coulter counter Model S Plus-II; CV = coefficient of variation (%); GA = glutaraldehyde; Hb = hemoglobin (mmol/l); Ht = hematocrit (%); LY% = lymphocyte percentage; LY# = lymphocyte counts; MCH = mean cell Hb (fmol); MCHC = mean cell Hb concentration (mmol/l); MCV = mean cell volume (μm^3 = fl); MPV = mean plt volume (μm^3 = fl); Pct = plateletcrit (%); PDW = plt distribution width; plt = platelets; PLT = plt counts (10^9/l); PMTR = parameter; (I)QC = (internal) quality control; rbc = red blood cells; RBC = rbc counts (10^{12}/l); RDW = rbc distribution width (%); wbc = white blood cells; WBC = wbc counts (10^9/l); $\bar{x}$ = mean value.

report we have described the preparation of a seven-parameter blood control of medium-term stability [4]. These 'seven' parameters were white blood cell counts (WBC), red blood cell counts (RBC), hemoglobin (Hb) and the four red blood cell (rbc) parameters mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and hematocrit (Ht). Additionally, red blood cell distribution width (RDW) was monitored [4].

In another paper [5] we described a human platelet(plt)-white blood cell (wbc) control for the Coulter counter Model S Plus-II (CSP-II). In this article we report our studies on the development of a multiparameter QC material for the CSP-II, combining the seven-parameter QC material with (fixed) human plt.

Materials and methods

Blood processing

Blood was routinely collected in a standard blood bag containing 75 ml of citrate phosphate dextrose-adenine (CPD-A). Plasma, wbc and plt were removed by centrifugation and sterile column filtration was performed as described before [4].

The sterile preservation medium containing wbc-substitutes and fixed human plt (for details see below) was added through a port in the bag using a standard connecting tube with two spikes. For rheological reasons a Ht equal to that of normal blood should be chosen, as it is recommended that control blood be as similar to patient blood as possible [6]: it is well known that Ht has a decisive influence on blood viscosity [7]. Spikes allow for faster blood flow rates than narrow gauge needles; the former should be preferred in order to speed up the blood processing and to avoid the application of considerable pressure and concomitant possible hemolysis due to too high shearing stresses [8].

Under occasional mixing portions of about 8 ml of the suspension were aseptically passed through the connecting tube (using a Kocher clamp) into 10-ml polyethene bottles (Tamson, Zoetermeer, The Netherlands. Order Nos. 420F 1 and 420F 11 (caps)) in a laminar flow cabinet. Although these bottles are not claimed to be sterile, blood cultures always proved to be sterile in a randomly chosen bottle filled with the control blood. (The plastic bags containing the bottles and caps were opened and closed only in the cabinet.) The filled bottles are stored in the refrigerator.

The preservation medium

Apart from the addition of fixed plt, the medium was essentially the same as the one described earlier [4]. The slight modifications involve:

(1) the EDTA-concentration was decreased from 15 mmol/l to 5 mmol/l for the reasons mentioned before concerning the Ht determination [4];

(2) 5 mmol/l adenosine was added because of its well-established benificial effect on membrane stability [9];

(3) the final K-concentration was higher by 10 mmol/l;

(4) the final pH of 7.9, instead of 8.5, was achieved with HCl instead of NaOH (tripotassium phosphate was used instead of dipotassium phosphate). (Modifications (3) and (4) are not essential.)

The exact composition of the final medium used is: 5 mmol/l $NaH_2PO_4 \cdot 2\ H_2O$, 30 mmol/l $K_3PO_4 \cdot 3\ H_2O$, 5 mmol/l K_2-EDTA $\cdot 2\ H_2O$, 5 mmol/l adenosine, 2.2% mannitol, 1 g/l gentamicin, 4 mol/l HCl to pH 7.9 (20 °C). After autoclaving at 120 °C for 30 min and cooling, the solutions or suspensions were added under sterile conditions (final concentrations in parentheses): albumin (3%), glucose (0.5%), Stabicells ($5–15 \times 10^9$/l) [4] and fixed human plt ($100–400 \times 10^9$/l). The final osmolality was set at approximately 300 mOsm/kg by the addition of sterile distilled water. The preservation medium can be kept for several months (we do not yet have sufficient experience of keeping fixed plt). It is important initially to make higher concentrations than stated by leaving out enough water to compensate for the volumes to be added after autoclaving.

A practical protocol for a final volume of 300 ml preservation medium is (M_r = relative molecular mass in parentheses): 145 ml distilled water, 235 mg $NaH_2PO_4 \cdot 2\ H_2O$ ($M_r = 156$), 2400 mg $K_3\ PO_4 \cdot 3\ H_2O$ ($M_r = 266.3$), 606 mg K_2-EDTA $\cdot 2\ H_2O$ ($M_r = 404.5$), 390 mg adenosine ($M_r = 267.3$), 33 ml mannitol 20%, 300 mg gentamicin, approximately 1.5 ml 4 mol/l HCl. After autoclaving and cooling: 45 ml albumin 20%, 15 ml glucose 10%, approximately 1 ml Stabicells, 20–60 ml fixed human plt, distilled water to 300 ml and a final osmolality of 290–310 mOsm/kg.

Platelet fixation

A plt concentrate, prepared from buffy-coats [5], was transferred to a sterile 100-ml glass injection bottle and diluted, if necessary, with ACD until a final concentration of approximately 2.10^{12} plt/l (for reasons of standardization). While gently swirling, 200 µl glutaraldehyde (puriss., 25% in water for electronmicroscopy, under argon, photosensitive — Fluka, Buchs, Switzerland, No. 49625 — store frozen, see 'Discussion') was carefully added. Fixation was allowed to proceed on a roller mixer for at least 2 h. The suspension should remain very fine; indeed no or very few aggregates should be seen on microscopic inspection. After fixation 20% albumin was added in a final concentration of approximately 3%. Depending on the plt concentration desired in the control, a certain volume of this fixed plt concentrate is added to the preservation medium.

Instructions for use

Before use, the bottle is carefully inverted by hand and allowed to warm to room temperature on a roller mixer. There is no need to return the bottle to the refrigerator. The bottle may be left ready to hand on a roller-mixer for 8 h or even longer (see 'Results'), facilitating frequent use [4,6].

Calibration and QC of the CSP-II

The calibration and QC of the CSP-II were basically performed as previously described [4], and the CSP-II was monitored by Bull's algorithm, as used in Coulter's QC computer program [10].

Results

Table I shows the results obtained for the eight primary parameters of a representative blood control preparation over several weeks. Since the derived parameters Ht, MCH, MCHC and plateletcrit (Pct) are calculated by the CSP-II, their values are dependent on those of the primary parameters and consequently they are not given. Table I shows non-significant differences between the values of the described control in the three test periods, indicating perfect stability of all the parameters during the entire test period. The Table also shows that the CV's of the described control are slightly higher than those of Hyland's eight-parameter control. It should be recalled, however, that Hyland's control was measured once every morning under standardized conditions, whereas the described control was used at random while rocking and rolling on a mixer for up to 8 h and sometimes even longer.

Fig. 1 reproduces the composite display of the 7-week-old control on the CSP-II. The arrow in the WBC-area points to a small shoulder due to plt aggregation. This shoulder is sometimes much larger in commercial controls. The LY% and LY# (counts) are not automatically printed out, but single aperture values can be recorded. This is shown in Table II for a different, representative control.

Table II also shows the excellent average intra-assay CV's of all the parameters ($n = 10$). Furthermore, this type of test enables one to judge the inter-aperture agreement. In this case highly discordant CV's of aperture 2 for WBC, LY% and

TABLE I

The means ($\bar{x}$) and coefficients of variation (CV's) of the first 20 measurements, those of all 49 measurements and those of the last 13 measurements for the 8 primary parameters of the control described and Hyland's 'normal' 8-parameter control

The non-significant differences between the mean values of the control described in the three periods indicate perfect stability of all the parameters in the entire 7-week test period. CV's of the described control are slightly worse than those of Hyland's 8-parameter control (see text).

	Control described						Hyland *	
	1st week ($n = 20$)		6th + 7th week ** ($n = 13$)		Total period ** ($n = 49$)		Total period ($n = 21$)	
	$\bar{x}$	CV	$\bar{x}$	CV	$\bar{x}$	CV	$\bar{x}$	CV
WBC	14.4	2.1	14.3	2.0	14.3	1.9	7.8	1.5
RBC	5.10	1.9	5.11	1.8	5.12	1.7	4.55	1.2
Hb	10.5	1.5	10.5	1.8	10.5	1.7	8.3	1.2
MCV	96.7	1.1	94.7	1.1	95.9	1.4	87.3	1.0
RDW	13.5	3.6	13.5	5.4 **	13.6	4.2 **	18.9	1.4
PLT	167	3.0	176	5.6 **	172	4.9 **	232	4.7
MPV	8.0	2.2	8.1	1.5	8.2	3.1	7.2	1.4
PDW	17.0	1.9	17.2	3.0 **	17.1	2.3 **	14.6	1.6

* Lot No. 838 YN.

** Including the results of a control that was mixed on a roller-mixer during 3 days (see Table III).

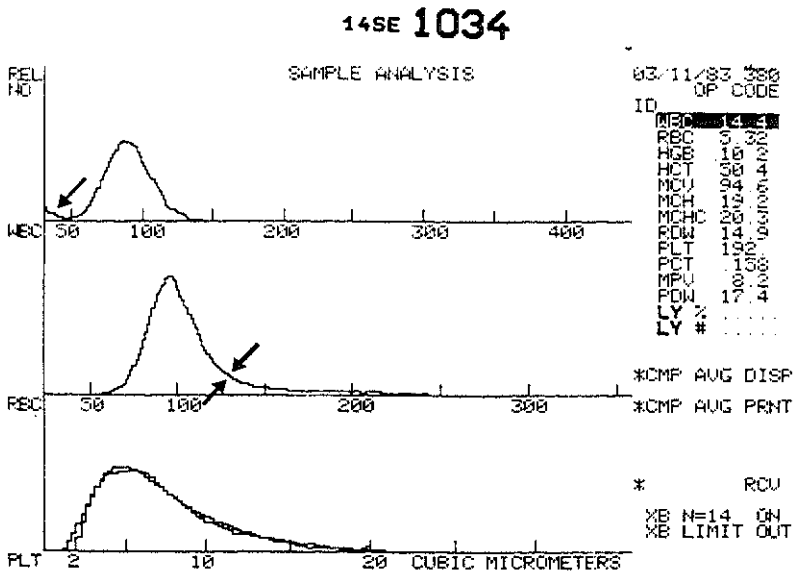

Fig. 1. Composite display of the 7-week-old control on the Coulter S Plus-II. The arrow in the WBC-area points to the relatively small shoulder due to platelet aggregation. The double arrow in the RBC-area indicates slight skewing to the right, most probably due to swelling of some red blood cells.

TABLE II

Intra-assay reproducibility of a representative control as generated in Coulter's QC program

Excellent (average) results are found. Single aperture (AP) values for LY% and LY# are displayed. The highly discordant CV's of AP 2 for WBC, LY% and LY# indicate aperture malfunction (***).

	Startup							
	Reproducibility/calibration				Date 26-10-83 OPR 123			
	Mean				CV			
PMTR *	AVG *	AP1 *	AP2	AP3	AVG	AP1	AP2	AP3
WBC	12.25	12.57	11.77	12.05	1.3	1.3	11.0 ***	1.7
RBC	3.952	3.931	3.982	3.944	1.2	1.4	1.5	1.1
HGB	8.12	8.12	8.12	8.12	1.1	1.1	1.1	1.1
HCT	37.21	36.93	37.55	37.15	1.4	1.7	2.1	1.1
MCV	94.1	93.9	94.3	94.1	.4	.8	.9	.7
MCH	20.51	20.63	20.37	20.56	.9	1.0	1.1	.7
MCHC	21.80	21.96	21.58	21.83	1.0	1.4	1.5	1.0
RDW	13.46	13.58	13.42	13.35	1.2	2.1	1.4	2.0
PLT	283.0	286.3	275.6	287.1	2.1	4.7	2.4	1.9
PCT	.2269	.2322	.2186	.2297	2.3	3.6	3.0	4.3
MPV	8.02	8.11	7.91	8.01	2.5	2.9	2.7	3.8
PDW	16.83	16.80	16.81	16.87	1.0	1.6	2.5	3.0
LY%	.0 **	74.9	71.3	75.9		.8	14.0 ***	1.4
LY#	.00 **	9.44	8.51	9.12		1.0	20.8 ***	2.8

$n = 10$.

* See list of abbreviations.

** Backlighted.

*** Aperture-2 malfunction.

TABLE III

Mechanical stability testing of the control

Comparison of means and CV's shows acceptable stability.

		n	WBC	RBC	Hb	MCV	RDW	PLT	MPV	PDW
After 5 min	mean	2	13.9	5.07	10.5	94.8	13.9	186	8.0	18.0
Total period	mean	6	14.1	5.15	10.4	94.4	14.3	180	8.1	17.5
(51 h)	CV(%)	6	2.1	2.3	1.2	0.5	4.3	6.9	1.6	3.6
After 51 h	mean	2	14.5	5.28	10.3	94.6	15.0	186	8.3	17.5
Inter-assay (Table I, 1st week)	CV(%)	20	2.1	1.9	1.5	1.1	3.6	3.0	2.2	1.9

LY# are found, indicating aperture malfunction (***).

Table III gives the results for the mechanical stability testing. In order to investigate mechanical resistance and stability, the 7-week-old control blood was allowed to come to room temperature and remain so over the total test period of 51 h. During daytime the control was rocked on a roller-mixer for 3 h on the first day and for 6 h on the second and third days. The Table shows that the total-period-CV's are generally comparable with those of the interassay CV's of Table I, indicating acceptable mechanical stability of the control. In fact, Fig. 1 shows the histogram of the '51-h-old' control, which is nearly identical to that of the 'fresh' control (not shown). The only departure from a fresh control pattern consists of a slight skewing to the right of the RBC-histogram (as indicated by double arrows in Fig. 1), which probably indicates swelling of some rbc. This may be reflected by an increased RDW (Table III). Slight deterioration of rbc-stability is also indicated by the fact that the micro-Ht of the '51-h-old' control was only 43.8% ($n = 2$), whereas that of the CSP-II was 48.7% ($n = 6$) and that of the freshly prepared control 7 weeks before was 49.1% ($n = 2$). The micro-Ht supernatant had a brown color due to hemolysis, but a sharp interface with the packed rbc was obtained (see 'Discussion').

Discussion

Mechanical stability testing

Mechanical stability testing is described to corroborate the statement made in the subsection 'instructions for use' that there is no need to return the bottle to the refrigerator. This enables the user to leave the control ready to hand on a roller-mixer, greatly facilitating frequent use (vide infra). As shown in the 'Results' slight deterioration of the control occurs. However, Table III demonstrates stable values as measured in a CSP-II. Clarification of this phenomenon is given in the discussion in one of our previous papers [4] under the subheading 'Constant Hb and rbc-values despite gradual oxidation and haemolysis'. (Our experience has shown that it is necessary to stress that this applies only to Coulter-type (resistive-particle) counters and consequently not to 'optical' instruments [11], which are sensitive to hemolysis.) Last but not least, it should be noted that the Ht of the described control is fairly high (50%, see Fig. 1). Hoegman et al [12] have shown that 'mechanical traumatiza-

tion (centrifugation or shaking) caused considerably more damage to red cells when they were highly concentrated than when they were diluted' and that 'this tendency was clear-cut, irrespective of how the red cells had been stored and in which medium they were suspended during shaking'. This explains why the hemolysis in the described control is higher than that in our previously described control with a Ht of only 26% [4].

Platelet fixation

In an earlier publication [5] we showed that plt-counts are stable for at least 5 months (in fact we later found a stability remained for at least 12 months) provided they were kept in a medium of low pH (5.5 at 20 °C). Unfortunately, many subsequent attempts to keep plt-counts constant failed when plt in ACD were added to rbc suspensions. One of the possible reasons is the higher pH's of the rbc-suspension, although plt-counts were generally constant for about 18 days. Many attempts to avoid aldehyde-fixation with its 'inherent' aggregation disadvantages [5] were made by adding several substances separately or in combination: (1) prostaglandin E_1, a well-known aggregation inhibitor [13,14]; (2) theophylline, a phosphodiesterase inhibitor, that prevents cyclic AMP breakdown [15]; (3) apyrase, which converts ADP, a potent aggregation inducer, which is released by rbc [16,17]; (4) taxol, a plt microtubule stabilizing agent, that was claimed to protect plt microtubules from disassembly in the cold and to preserve the discoid shape of most plt [19]. None of these substances solved the problem of plt swelling and instability.

Consequently, aldehyde fixation seems necessary to obtain stable plt. Eventually we found that 'inherent' aggregation, the main problem of plt fixation, can be almost completely prevented by fixation at a lower pH. We used ACD with a pH of approximately 5.5 (20 °C). The fixation was carried out with colorless glutaraldehyde (GA) that had been stored in the freezer (approximately −20 °C) for several years to slow down GA polymer formation [20]. The possible importance of using GA solutions of high purity has been demonstrated by data indicating that the monomeric and polymeric forms of GA do not affect tissue in the same way [21]. The basis for these differences has not yet been fully clarified [21]. We used the ratio of the solution's absorbances at 280 and 235 nm (polymers) to assess the purity of the GA [20]. We found a ratio of 0.30 in a 0.5% dilution in water. Knowledge of the purity of GA used might be important to reproduce the described fixation process.

Fig. 2 shows a histogram of a 1 : 2 dilution in ACD of a fixed plt suspension. This histogram reveals that at pH 5.5 aggregation is indeed almost completely prevented. This is demonstrated by the relatively small shoulders in the wbc and rbc areas and by the fairly low apparent wbc, rbc and Hb-values. This is further substantiated by the data given in Table IV showing non-significant differences ($p > 0.05$) between plt-counts in fixed and in non-fixed plt suspensions.

The major advantage of low-pH fixation is that it enables unprecedented high concentrations of plt ($2–8.10^{12}$ plt/l) to be fixed without substantial aggregation [5,22–23]. This greatly facilitates the preparation of the multiparameter control. Studies are in progress to optimize further the fixation conditions in order to try and completely prevent aggregation.

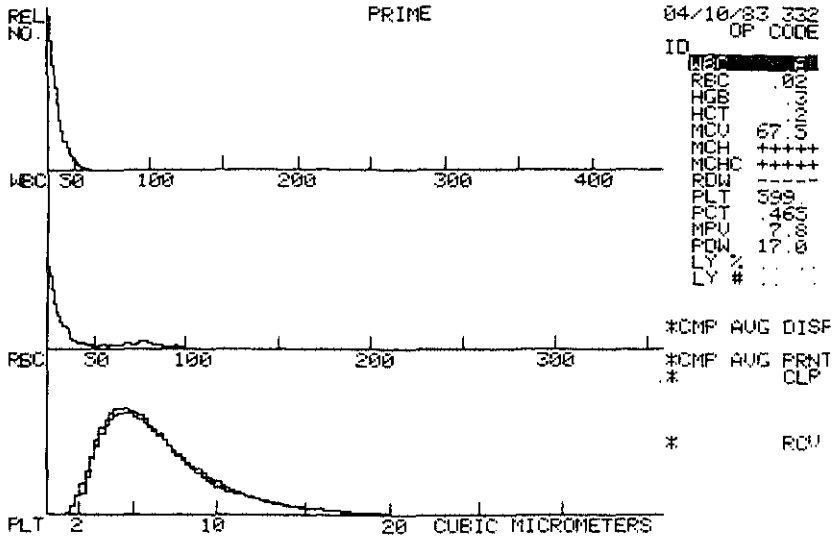

Fig. 2. Histogram of the 1 : 2 dilution of fixed platelets with acid citrate dextrose (pH approximately 5.5 at 20 °C).

Optimization of internal quality control (IQC)

The objectives of this paper are

(1) to describe a simple protocol for the fairly inexpensive preparation of a QC material for Coulter-type blood cell counters;

(2) to provide QC blood in sufficient quantities to optimize IQC. This QC material can basically contribute in several ways to optimization of IQC, preferably in conjunction with the use of some computerized 'average of normals' program [4,10]. Firstly, the availability of a fairly inexpensive, independent source of cell control of medium-term stability makes it possible to check the 'primary calibration material' for any deterioration or vial variances [4]. Secondly, by frequently monitoring drift and other gradual malfunctions and promptly tracing any abrupt instrument failures [4]. Last but not least, to contribute basically to the application of Shewhart's pioneering, brilliant IQC philosophy: to differentiate 'some stable system of inherent chance causes' from 'assignable causes of error' [24–26]. In other words,

TABLE IV

Relevant mean data ($n = 3$) of fixed and non-fixed platelet suspensions both diluted 1 : 2 in ACD

Note the non-significant differences between the PLT and the significantly higher apparent WBC- and Hb-values. For comments see text.

	WBC	RBC	Hb	PLT	MPV	PDW
Fixed	0.8	0.01	0.3	588	7.7	17.0
Non-fixed	0.2	0.02	0.1	603	8.1	17.3

these materials serve three basic purposes of IQC:

(a) Process control, to be executed 'ad hoc' by the technician by regularly running the material and instantly judging the results obtained.

(b) Retrospective IQC by the hematologist or the 'Q.C. officer' by evaluation of the proficiency attained both within-day and between-day (Shewhart charting) [26].

(c) Blind controls to assure optimal IQC. This is done by regularly interspersing and evaluating results of other preparations of known composition, disguised as routine patient specimens. The tendency to delegate this type of control to external quality assessment schemes threatens the alertness and readiness of action of the laboratory [27].

In short, these materials are intended to contribute to the essence of IQC: continuous self-auditing and continuous attempts at improvement of performance.

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Outdated blood and redundant buffy-coats as sources for the preparation of multiparameter controls for Coulter-type (resistive-particle) hemocytometry

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Key words: Buffy-coats; Ghosts; Hemolysis; Internal quality control; Multiparameter hemocytometry control; Outdated blood; Resistive-particle counters

Summary

Outdated, buffy-coat depleted, CPDA-1 blood and redundant buffy-coats were used as sources for the laboratory preparation of controls for Coulter-type (resistive-particle) hemocytometry. Deteriorated white blood cells and platelets and potentially interfering microaggregates with volumes not exceeding 400 fl are shown to be virtually completely removed by centrifugation and filtration. Addition of fixed red blood cells as white blood cell substitutes and of isolated, fixed platelets enable the preparation of multiparameter controls of short-to-medium-term stability. The availability of these simple, inexpensive controls can contribute significantly to optimal internal quality control in hemocytometry.

Introduction

In our previous papers on the laboratory preparation of hemocytometry controls [1,2] fresh blood was always used as the starting material. However, several considerations prompted us to study the potential of using outdated, buffy-coat depleted blood as the source for these controls. Moreover, the addition of platelets, isolated from buffy-coats and fixed as previously described [2,3] allows us to produce

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multiparameter controls of short-to-medium-term stability. This is demonstrated by measurements in a Coulter Counter Model S-Plus II, equipped with a (standard) Coulter Video Data Terminal (VDT) and matrix plotter [4]. The low cost and easy availability of these starting materials together with the simple methods of preparation of these controls enable laboratories with Coulter-type (resistive-particle) counters to use these controls frequently along the lines previously described [2], thus contributing to internal quality control [2].

Materials and methods

Centrifugation and filtration of outdated, buffy-coat depleted blood

Outdated, buffy-coat depleted, CPDA-1 blood, as routinely stored in The Hague's blood bank, is used as one of the starting materials for our controls. After centrifugation in a Christ 8.6 or 6.4 S centrifuge at 22°C for 15 min at about $4{,}200 \times g$, the supernatant plasma, containing most of the remaining buffy-coat and probably also most of the formed microaggregates [5], is pressed out in a plasma extractor [1,2]. The red blood cells (rbc) are filtered under sterile conditions through a cotton-wool filter column to remove the great majority of the remaining white blood cells (wbc), the platelets (plt) and probably also the microaggregates (microaggr) [1,2]. All these procedures are performed with simple, routine blood bank tools and methods. After the blood has been processed in this way, the bag is weighed (the weight of empty bags being known) to estimate the volume of the blood. Moreover, after mixing, a sample is run through the Coulter Counter to evaluate whether the wbc, plt and small microaggr are sufficiently removed and to determine the hematocrit (Ht). From the total volume and the Ht the volume of the wash (saline) solution, which may vary in different filtrates, in the rbc suspension is calculated. In previous papers [1,2], we failed to stress the importance of this volume for the calculation of the amount of mannitol to be added in order to arrive at the appropriate final mannitol concentration.

Preparation of the controls

Fixation of platelets, harvested from fresh buffy-coats Isolation and fixation of plt in high concentration ($2\text{–}8 \times 10^{12}$/l plt) were performed, exactly as previously described [2].

Artificial preservation medium The same high-K, low-Na medium as previously described [2] (including fixed human plt) was used. The amount of mannitol to be added was calculated, taking into account the volume of the remaining saline solution, in order to assure a final mannitol concentration of 1.6%. In one of our previous papers [1], we mentioned a final concentration of approximately 2.2% as optimal for achieving a constant MCV. To prevent possible confusion, we wish to draw attention to the fact that in that paper we gave the concentration of mannitol in the preservation medium, not taking into account the volume of remaining saline in the filtered rbc suspension to be added to the preservation medium; generally the Ht of the filtered rbc suspension amounts to approximately 70%.

Compatible CPD(A) plasma As an alternative, compatible stored CPDA-1 plasma or factor VIII-depleted plasma was sometimes used instead of the artificial preservation medium. Stabicells (ca. 0.3 ml/100 ml) [1,6], gentamicin (some 400 μg/l or more) [1] and fixed plt [2] were added through a port into the plasma bag. Moreover, mannitol was added, again taking into account the volume of the remaining saline of the rbc suspension to be added, to achieve a final mannitol concentration of 0.55%. Since both citrate and mannitol are impermeable to rbc [1] and consequently both exert an oncotic effect [1], the required final mannitol concentration is lower than that in the artificial preservation medium. Notice, however, that the citrate concentration in the various anticoagulants varies substantially [7].

Actual preparation of the control The actual combining of the filtered rbc suspension with either the preservation medium or with compatible plasma as well as the filling of the control bottles were performed in exactly the same way as recently described [2].

Calibration and quality control of the Coulter Counter

Calibration and quality control of the Coulter Counter Model S-Plus II were performed as previously published [2].

Instructions for use

The instructions for use are the same as previously described [2]. It might be useful, however, to reiterate that the control may be left ready to hand on a roller mixer for 8 h or even longer (depending on its quality and age), facilitating frequent use.

Contraves

A Contraves Haemocell 400 H, coupled to a Contraves Digicell 3100 H, as well as a Dilutor 4020 were used (Contraves AG, Zurich, Switzerland).

Results

Figure 1 shows a facsimile of a matrix plot of a typical VDT-display of outdated, in this case 4-wk-old, buffy-coat-depleted, CPDA-1 blood after centrifugation and filtration. Note that the Ht (Hct) is 64.7%. The successful removal of wbc, plt and relevant microaggr by the described procedure is amply supported by the following facts: (1) the wbc and plt counts are virtually zero and the corresponding histogram areas are practically 'clean'; (2) the rbc histogram is fairly normal (remember that no or very few rbc-coating proteins are present [1]); (3) the histograms exhibit no evidence of remaining microaggr of interfering size.

Table I shows the results of the primary parameters of a representative control prepared in the described artificial preservation medium as measured in a Coulter S-Plus II and in a Contraves blood cell counter. The counters were not cross-calibrated. The measuring periods were 7 wk (November–December 1983) and 3 wk (December 1983) for the Coulter and Contraves counters, respectively. The table

TABLE I

Mean ($\bar{x}$) and CV for the primary parameters of a representative control prepared in the described artificial preservation medium as measured in two resistive-particle counters

The counters were not cross-calibrated. The measuring periods were 7 and 3 wk for the Coulter and Contraves counters, respectively. The outdated blood was 4 wk-old before preparation. Values for the commercial control 'Hyland eight-parameter Normal', measured in the Coulter Counter once every morning during approximately the same period, are added for comparison.

Counter		WBC	RBC	Hb	MCV	RDW	PLT	MPV	PDW
Coulter S-Plus II	$\bar{x}$	7.55	3.77	7.18	91.8	15.2	122	7.3	17.8
$n = 117$ (7 wk)	CV (%)	2.2	2.1	1.1	0.6	3.1	4.5	1.6	2.8
Contraves $n = 26–29$	$\bar{x}$	8.20	4.06	7.60	93.7				
(3 wk)	CV (%)	5.3 *	2.2	1.6	1.1				
Hyland's 'Normal'	$\bar{x}$	7.3	4.38	7.79	86.0	19.6	215	7.2	14.9
control (Coulter Counter)	CV (%)	2.5	1.3	1.5	0.4	1.3	5.2	1.2	1.0
$n = 31$									

* Upward trend with time.

exhibits CV values comparable to those described for fresh blood in one of our previous papers [2]. Furthermore, the table contains values for the commercial control 'Hyland eight-parameter Normal', measured in the Coulter Counter once every morning during the period mid-November–end December 1983 ($n = 31$). The

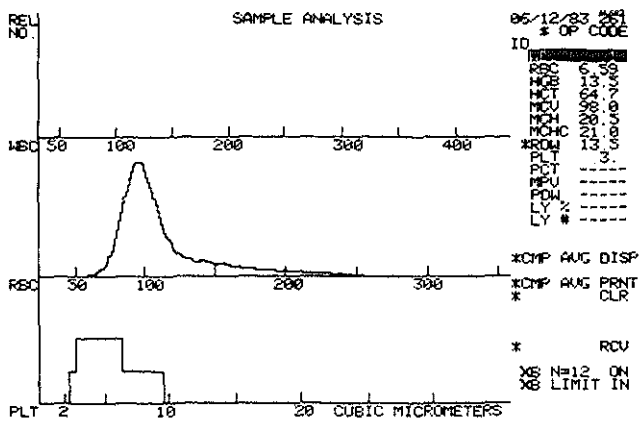

Fig. 1. Facsimile of a matrix plot of a typical Video Data Terminal display of outdated (in this case 4-wk old), buffy-coat-depleted, CPDA-1 blood after centrifugation and filtration. For comments see the text.

table also shows acceptable CV values (apart from that for WBC, which underwent an upward trend) for the control in a Contraves counter.

Figure 2 represents a time-based collage of some histogram plots of the control. The figure shows comparable plots for A (0 wk) and B (7 wk after preparation), although the rbc histogram in Fig. 2B exhibits the first signs of rbc swelling (arrow). However, Fig. 2B was made after 6 h of rocking and rolling on a mixer, showing

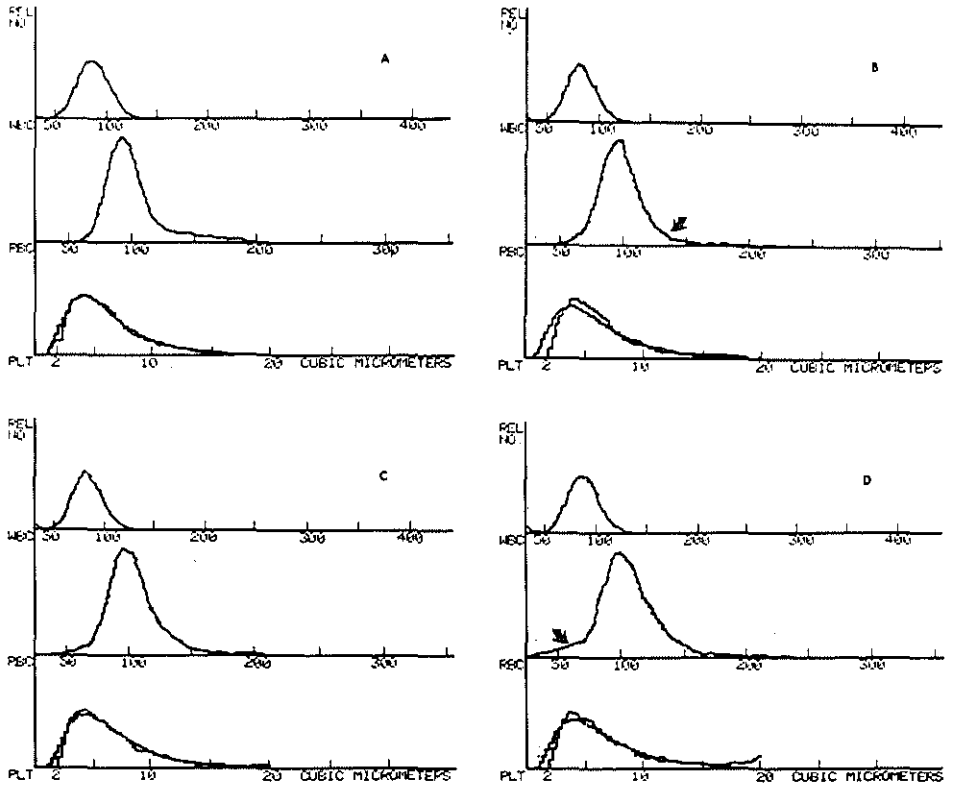

Fig. 2. Reduced collage of four histograms (A–D) of the control, suspended in the artificial medium (cf. Table I), plotted at different stages of the 4-mth observation period. The ages (wk) after preparation and the primary parameters at these ages of the control are as follows:

Plot	Age	WBC	RBC	Hb	MCV	RDW	PLT	MPV	PDW
A	0	7.4	3.63	7.2	92.4	14.5	120	7.1	17.7
B	7	7.1	3.68	7.0	91.7	15.5	119	7.4	19.0
C	10	8.1	3.97	7.4	95.3	16.2	122	7.6	17.5
D	16	7.5	3.73	6.9	96.0	17.8	128	7.4	18.4

Note that WBC, RBC and PLT are stable throughout the period, although in stage D minor departures from log normal PLT distribution were sometimes found, in contrast to the case shown in plot D, impeding the generation of values for mean PLT volume (MPV) and PLT distribution width (PDW). For further interpretations see the text.

TABLE II

Mean ($\bar{x}$) and CV for the primary parameters of a representative control prepared in 0.55% mannitol-containing, compatible CPDA-1 plasma as measured in two resistive-particle counters

The counters were not cross-calibrated. The measuring periods were 6 and 4 wk for the Coulter and Contraves counters, respectively The outdated blood was 4-wk-old before preparation. Values for Hyland's control were of the same order of magnitude as in Table I.

Counter		WBC	RBC	Hb	MCV	RDW	PLT	MPV	PDW
Coulter S-Plus II	$\bar{x}$	6.35	3.98	8.20	96.9 *	13.1 *	275	8.4	17.4
$n = 127$ (6 wk)	CV (%)	2.8	1.6	1.8	0.9 *	4.1 *	4.5	1.8	1.0
Contraves $n = 34$	$\bar{x}$	6.88	4.22	8.49	97.8				
(4 wk)	CV (%)	5.2 **	3.0	2.1	1.3				

* Slight, gradual increase with time.
** Upward trend with time.

fairly good mechanical stability of the control. The constancy of the rbc parameters MCV and RDW are only acceptable for about 7 wk. Notice several gradual changes occurring during the 4-mth-period: (1) MCV (after 7 wk) and RDW are increasing; (2) The right rbc-tail is decreasing, indicating diminution of 'cell coincidence, aperture artefact, doublets, triplets and/or agglutinates' [4]; (3) Skewing to the right is increasing due to the rbc swelling; (4) Skewing to the left (most pronounced in Fig. 2D; arrow) due to ghost (hemolyzed rbc) formation. As we pointed out before [1], hemolysis gradually increases leading to rbc ghosts which give MCV values mainly in the 50–70 fl range (depending on the salt concentration), at least under

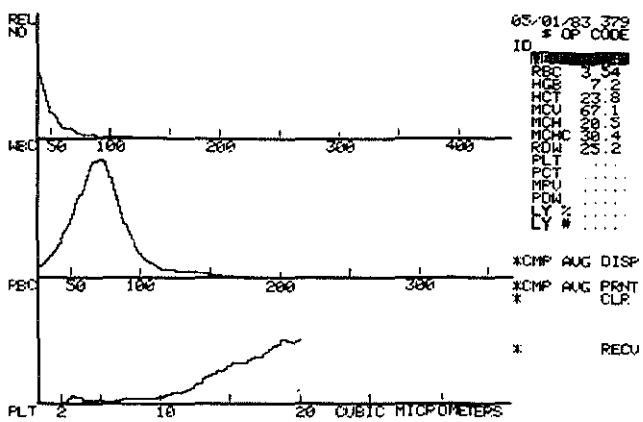

Fig. 3. Histogram of a water-induced red blood cell hemolyzate.

the geometric and electric current circumstances in the measuring apertures of the Coulter S-Plus II [1, 8]. This is demonstrated in Fig. 3 which shows the histogram of water-induced ghosts. We found extreme constancy of such ghost histograms and of their apparent MCV and rbc counts for a period of over 2 yr (not shown). This finding and the fact that 'pulses from the rbc bath representing cells 36 μm^3 and greater are classified as red cells' [4] explain why hemolysis most probably does not affect rbc counts during a long period.

Table II compiles the results of a representative control prepared in CPDA-1 plasma, containing 0.55% mannitol. The CV values are largely comparable with those of another control, prepared in our artificial preservation medium as shown in Table I. Both MCV and RDW exhibit a gradual, albeit slight, increase with time, possibly indicating that the mannitol concentration should be slightly increased. Again the table shows acceptable (except for WBC) CV values in the Contraves counter.

Discussion

Objections of The Hague blood bank to using fresh blood for the routine, medium-scale preparation of hemocytometry controls (in contrast to usage for small-scale experimental goals) as well as the fact that a number of blood bags inevitably get outdated in any blood bank (approximately 2% in The Hague's blood bank, yielding several hundreds of bags per year), prompted us to investigate the potentials of using outdated blood as the source for the preparation of our controls. For a clear appreciation of the matter it seems useful to recall that blood donations are performed on a voluntary basis in several countries, including Canada, Great Britain, France and The Netherlands; consequently fresh blood cannot be simply bought [9].

Outdated bank blood was successfully employed in one of our laboratories for some eight years, allowing for the use of unlimited volumes of inexpensive quality control material for the Coulter Counter Model S. Several disadvantages of the use of outdated blood have been previously mentioned [1]. The major difficulties included MCV problems and, especially for so-called 8-parameter flow hemocytometers, interference of microaggr and cell fragments in the wbc and plt range.

In this paper, the MCV problem was shown to be partly overcome by the addition of an appropriate amount of mannitol. Although we did not yet systematically investigate the issue, it is hard to avoid the impression from numerous experiments that MCV constancy in the artificial medium is superior to that in mannitol-containing plasma.

Microaggr with volumes ranging from 2 to far over 1,000 fl, consisting mainly of aged plt, wbc and strands of fibrin, are known to develop in blood during storage at 4° C [5,10–13]. However, their formation will be prevented for the greater part when the concentration of plt and wbc is low at the beginning of storage [14]. On average, $< 20\%$ of the plt and $< 40\%$ of the wbc remain in 'buffy-coat-free' rbc concentrate [14]. As described, our starting material consisted of buffy-coat depleted blood. We generally found $1–3 \times 10^9/l$ particles in the wbc-range and some $100 \times 10^9/l$

particles in the plt-range in such blood after 4–8 wk of storage (not shown). Further removal of microaggr from stored blood was recently described by Linko [5]. He found that centrifugation (5 min, 6,900 × g) and filtration of blood (stored > 1 wk) through a standard 170 μm transfusion filter removed most of the microaggr. We found excellent results after centrifugation for 15 min at 4,200 × g, removal of the plasma and part of the remaining 'buffy-coat' and filtration through a standard cotton-wool filter column, at least for microaggr with volumes not exceeding 400 fl, as is shown in Fig. 1. Linko [5] showed that 'centrifugation caused the small aggregates to firmly adhere to each other and form larger ones so that aggregates ranging from 10 to 80 μm virtually disappeared'. Microaggr with volumes exceeding 400 fl will not interfere with the Coulter measurements. Furthermore, in practice we never experienced any increased tendency to clogging or increased protein build-up of the measuring apertures due to high-volume microaggr, if any. So microaggr > 400 fl are likely to be largely or totally removed together with the plasma and buffy-coat after centrifugation and/or during the filtration process.

Stored packed cells (Ht approximately 80%) could also be used as starting materials. However, they are known to be inferior to buffy-coat-depleted blood (with normal Ht) because of their greater sensitivity to mechanical damage and hemolysis during storage and handling [15].

As Fig. 2 shows the addition of plt, isolated from buffy-coats and fixed as previously described [2,3], enable us to produce multiparameter controls with virtually complete absence of a shoulder in the wbc area, indicating that plt aggregation is almost completely [1,16] prevented, thus yielding multiparameter controls of short-to-medium-term stability. It should be emphasized, however, that this applies only to a lesser degree to instruments making use of optical measurements, as these are sensitive to hemolysis, causing decreasing rbc counts [17] and increasing artefacts in the plt-range (own experiments, not shown).

The results obtained by measurements in a Contraves counter show that the control is suitable for intralaboratory quality control in another resistive-particle counter, although substantial differences in the mean values of several parameters as measured in both instruments were found. It should be recalled, however, that the instruments were not cross-calibrated. We are currently investigating whether the control is suitable for low-budget, regional quality control cooperation and/or for interinstrument comparability studies.

Prevention of ethical objections, if any, to the use of fresh blood, low cost, the simple methods of preparation as well as the easy availability of the starting materials are the main assets of the described procedure. Large-scale intralaboratory implementation of these controls is bound to improve dramatically internal quality control in hemocytometry.

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Contraves values. The authors are also grateful to Dr. J.A. van der Does, Director of the regional blood bank 's-Gravenhage en Omstreken for valuable discussions.

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3.1.

A simple, inexpensive quality control material for Ortho ELT-8 platelet counts

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SUMMARY Dilutions of 1 : 600 and 1 : 3000 of 1 μm diameter polystyrene latex suspensions are simple, inexpensive materials for quality control of Ortho ELT-8 platelet counts. The coefficient of variation (CV) for a 12-week period at the 350×10^9/l level was 2·2%, comparable with that of a commercial control. The CV at the 70×10^9/l level was 3·5%.

The automation of platelet counting is essential in laboratories that analyse a large number of samples. However, accuracy and precision of the automated platelet count are continual challenges and even more difficult to achieve than for the erythrocyte or leucocyte counts.[1–3]

The ELT-8 (Ortho Diagnostics, Westwood, Ma 02090, USA) is a fully automated haematology instrument which utilises 100 μl quantities of blood to determine the standard seven-parameter blood count plus platelet (PLT) count. Cell-counting is based on the principles of laminar flow hydrodynamic centering of cells before one-by-one counting using a helium-neon laser optics system. Real-time relative-size frequency distribution curves (histograms) for red blood cells, white blood cells, and PLT can be seen on the CRT display screen.[4–5]

The proper method of calibrating a platelet or multiparameter counter is to perform multiple manual platelet counts with a phase-contrast microscope.[1,2,6] The calibrating medium should be fresh human blood, preferably having high and low counts. Once properly calibrated, instrument drift can be monitored with a stable suspension of platelet control or latex spheres.[1–3] Many expensive platelet reference materials are on the market.[3] Satisfactory 8-parameter quality control materials for the ELT-8 do not exist.[5]

In this paper the preparation of simple 1 μm diameter polystyrene latex sphere suspensions and their use as platelet controls in the ELT-8 instrument are described.

Method and results

A vial with a 2·5 ml suspension of Dow latex microspheres, diameter 1·091 μm (SD 0·0082 μm), was obtained from Serva (Serva Feinbiochemica, D 6900 Heidelberg, West Germany). The approximate dry weight is 10%, d(20°C) = 1·05 and n_D (20°) = 1·59 (polystyrene). The price is approximately 150 DM. After vortex-mixing, 20 μl of the latex suspension was added to 12·0 ml Isolac (Ortho) and 0·200 ml gentamicin (10 mg/ml): *normal* 1 μm latex suspension. The *low* 1 μm latex suspension was prepared by adding 8·0 ml Isolac and 0·10 ml gentamicin to 2·0 ml of the normal 1 μm suspension. The suspensions were at 4°C or at room temperature during storage and/or measurement; they were measured after manual mixing, air bubbles having been cleared before aspiration or after mixing on a roller mixer. The suspensions were periodically measured over a period of 12 weeks, during which the performance of the instrument was monitored with the commercial platelet control Quanticel® Normal (BHP Inc, West Chester, PA, USA) and was found to be satisfactory provided the ELT-8 was occasionally recalibrated (Table). The Table shows comparable

Results of platelet measurements in ELT-8 of Quanticel Normal, normal and low 1 μm latex suspensions. Total period 12 weeks.

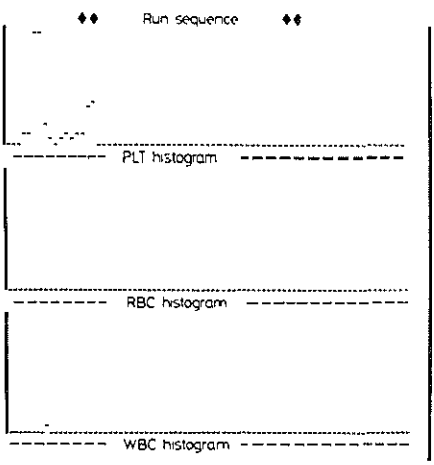
		n	$\bar{x}$ $\times 10^9$/l	*SD*	*CV* (%)
Quanticel normal	Total period	28	299·3 (260–300*)	6·7	2·2
Latex normal	First 7 days	10	345·0	11·1	3·2
	Total period	57	345·9	7·4	2·2
	Last 10 days	10	341·1	4·8	1·4
Latex low	First 7 days	10	69·2	3·8	5·4
	Total period	54	70·0	2·5	3·5
	Last 10 days	10	69·0	2·0	2·9

*Stated range.

CVs for Quanticel Normal and the normal latex suspension. The Table also shows good linearity for the normal and low latex suspension (calculated value for the low suspension 2·0/10·1 × 345·9 = 68·5). As can be seen in the Table, no significant differences exist between the mean counts of the first 10, the last 10, and the total number of measurements of both the normal and the low latex suspensions. The greater imprecision in the first seven days compared to that in the other periods was caused by slightly too high values for both Quanticel and the latex suspensions during days 6 and 7. The instrument was therefore cleaned and recalibrated.

Discussion

The Table shows the excellent performance of the 1 µm latex suspensions as controls for ELT-8 platelet measurement. Costs are negligible (dilutions of 1 : 600 and 1 : 3000) compared with those of commercial controls. The PLT histogram shows two peaks approximately covering a normal native PLT distribution curve (Figure).



Histogram displays of normal 1 µm latex suspension.

The response of a light-scattering sensor in a flow instrument is not a linear function of particle volume or diameter because of the strong dependence of such a response on the index of refraction, optical density, surface structure, and other optical properties of the particle.[7,8] This is clearly demonstrated here; 1 µm diameter latex beads, having an approximate volume of 0·5 fl, show approximately the same response as human platelets with a mean volume of 7·0–10·5 fl.[9] The index of refraction of latex is 1·593, whereas that of live cells is approximately 1·374.[8] Obviously latex sphere suspensions cannot be used for platelet volume calibration in the ELT-8 but they are shown to be simple, inexpensive quality control materials for ELT-8 platelet measurements.

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3.2.

Sterile, Medium Scale Age Fractionation of Human Red Blood Cells

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After sterile removal of white blood cells and the majority of platelets from blood in a regular transfusion bag, the remaining red blood cells were separated under sterile conditions into a relatively young and a relatively old fraction. Making use of the different densities of old and young red blood cells, the separation occurs in a double transfusion bag by centrifugation, after the cells have been carefully layered on top of Percoll® of "tailor-made" density.

The successful results of this simple and inexpensive technique are assessed by comparing the creatine concentration and the acetylcholinesterase activity in both fractions.

Thus, this medium scale fractionation technique yields hundreds of ml of sterile, young red blood cells.

Keywords: Medium scale fractionation; Percoll®, red blood cell fractionation; sterile fractionation.

Introduction

In the course of investigations into the laboratory preparation of quality control materials for flow haemocytometry [1], we were in need of a method for the sterile separation of hundreds of ml of young red blood cells (rbc). Perusal of the literature revealed only one medium or large scale method (hundreds or thousands of ml) for rbc fractionation, using the recently introduced blood cell separators [2–4]. These cell processors are, however, expensive, not universally present and primarily developed and in use for in vivo apheresis techniques [2–4].

The objective of this paper is to describe our development and evaluation of an alternative method for medium scale fractionation of rbc, employing simple means. After separation of the buffy-coat in a plasma extractor [5], white blood cells (wbc) and the majority of platelets (plt) are removed by the well-established routine method of sterile cotton-wool filtration [6]. Making use of the different densities of old and young rbc [7, 8], rbc fractionation is performed in a double transfusion bag by centrifugation, after blood has been carefully layered on top of Percoll® of a "tailor-made" density.

Materials and Methods

Preparation of red blood cell suspension for fractionation from citrated whole blood

In this paper we shall describe the whole blood processing procedure for *one* normal 500 ml ACD or CPD blood unit. It will be evident that scaling up can be achieved by simultaneously processing several blood units.

After routine centrifugation of the regular double blood bag and pressing out the plasma and buffy-coat in a plasma extractor [5], the remainder of wbc and plt are removed by filtration through a sterile cotton-wool column. We used the Cellselect leukocyte filterset D from the NPBI (Nederlands Produktielaboratorium voor Bloedtransfusie-apparatuur en Infusievloeistoffen), P. O. Box 9148, 1006 AC Amsterdam (Order number 1004). This routine technique has been shown [6] to be the method of choice for the preparation of wbc-free and plt-poor rbc suspensions.

After centrifugation and removal of the saline via the satellite bag in a plasma extractor [5], a representative blood cell sample is drawn without using the ports of the primary bag. This is done by entirely clamping the empty satellite bag at about one tenth from the top, leaving the headspace in connection with the primary bag via the transfer tubes and by carefully homogenizing the suspension in these compartments. The sample, obtained through one of the ports of the satellite bag, is to be used to determine the exact Percoll density needed for a successful fractionation (see below).

Preparation of Percoll®

Percoll®, a colloidal silica coated with polyvinylpyrrolidone, was purchased from Pharmacia Fine Chemicals, Sweden, in a sterile 1 L unit (cost about $ 70). Percoll was divided into quantities sufficient for 1 blood bag by filling out 225 ml into four 300-ml glass injection bottles each. After the addition of 17 ml of distilled water the Percoll was sterilized for 20 min at 120 °C. Since Percoll has a low osmolality (20 mOsm/kg) and autoclaving with salts causes gelation, sterile saline solutions must be added after sterilization. To minimize the volume of salt solutions to be added afterwards, 17 ml of distilled water was added before sterilization and 8.3 ml of 27% sterile sodium chloride solution was added after sterilization, yielding an osmolality of about 300 mOsm/kg. These solutions are stable at room temperature. To achieve the approximate density of rbc (1.080–1.115) [9] some 38 ml of sterile 0.9% saline are supplemented yielding a working Percoll solution with a density of about 1.105. This density can easily be determined by refractometry, as indicated in the Percoll package insert. The exact density needed is dependent on (1) the density of the rbc to be fractionated and (2) the amount of remaining saline after the majority of the saline had been removed after filtration of the blood through the cotton-wool column, as well as on (3) the desired ratio of the fractions of relatively old and relatively young rbc.

Assessment of the desired Percoll density

By the addition of increasing, exactly known amounts of extra saline, a series of test tubes of decreasing Percoll densities is prepared. After carefully layering aliquots of the sample rbc suspension (see above) on top of the Percoll solutions and after centrifugation, preferably under the same conditions as the actual large-scale centrifugation, the desired density can be assessed. Thus, the calculated amount of extra saline can be added to the working Percoll solution (generally about 15 ml).

Age fractionation

By using 2 large, long-pronged clamps and 2 additional ordinary Kocher clamps, the red cell suspension is pressed into the bottom compartment of the primary blood bag, as shown in Fig. 1. Via one of the ports of the satellite bag, Percoll of the appropriate density can be layered on top of the rbc without using the

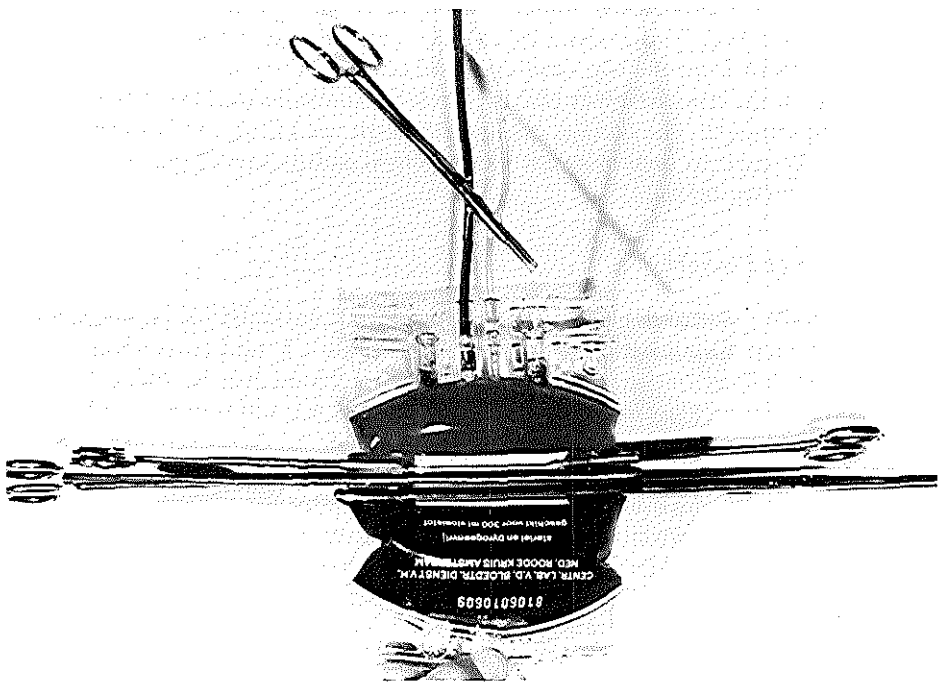

Fig. 1. Careful layering of Percoll® of appropriate density on top of red blood cells, using several large, long-pronged clamps. The Percoll was added via the satellite bag and one of the connecting tubes (light colour), obviating the need for using the injection ports. Since the subsequent centrifugation occurs with the ports in downward position, they may not be used before centrifugation, to prevent leakage. The other connecting tube shown (dark colour) was used to take a representative red blood cell-saline sample for the preliminary accurate density assessment

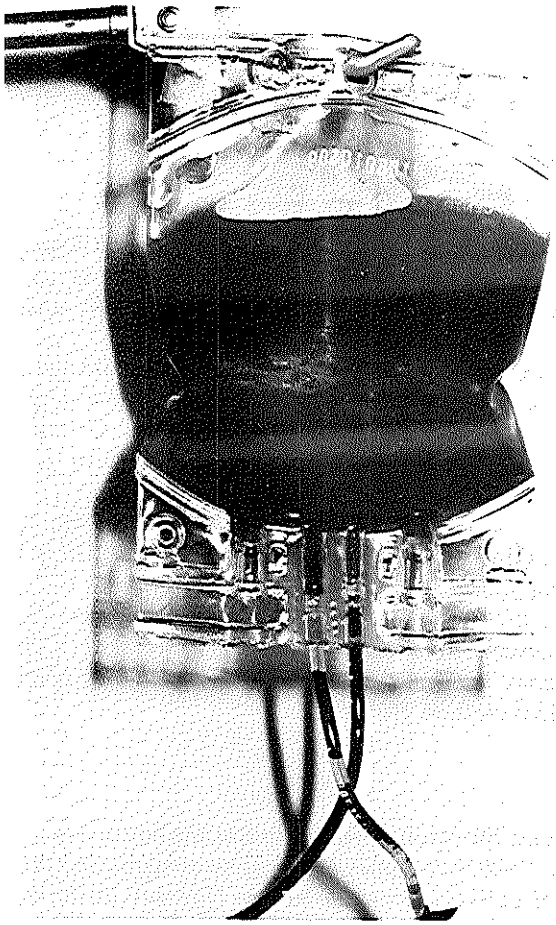

Fig. 2. Typical result of the fractionation after centrifugation. By chemical means, as described in the text, the lower red blood cell (rbc) layer is shown to consist of comparatively old rbc, the upper layer of the comparatively young rbc

ports of the primary bag. After sealing the connecting tubes (not too close to the primary bag) the satellite bag can be discarded. After inversion of the primary bag and careful removal of the clamps, the rbc are now on top of the Percoll. The bag is carefully placed upside down in a centrifuge beaker, that subsequently is stuffed up with rubber and plastic pads to prop the bag during centrifugation. This spinning occurs at routine bloodbank conditions, leakage being impossible because the ports are intact. Figure 2 shows 2 distinct (dark) cell layers after centrifugation, the bottom layer consisting of the relatively old fraction.

Through one of the ports of the primary bag, the layers can consecutively be harvested and washed with saline in separate bags. Since the density of the Percoll is greatly decreased by the addition of saline, the washing should be performed with

the bags in the normal upright position, the cells being in the bottom layer after centrifugation. The saline can be pressed out in a plasma extractor and washing can be repeated.

Tests of age fractionation

The content or activity of several substances in the rbc decrease with increasing rbc age [7–8]. Of the many age indicators described we chose creatine and acetylcholinesterase (ACh-ase) (EC 3.1.1.7) because of their stability and discriminating power [7, 10].

There is a non-linear relationship between rbc age and the creatine content [10]. The creatine concentration in young rbc is considerably higher than in older rbc. Creatine was assayed by the colorimetric method of Griffiths [11] as modified by Li et al. [12]. The intra-assay coefficient of variation (CV) (n = 10) was 3.4%.

ACh-ase activity in the rbc membrane decreases with age. The activity was determined according to the slightly modified method of Michel [13, 14]. In this method the pH decrease due to acetic acid formation from the substrate acetylcholine is a measure of ACh-ase activity. The modification consists of accurately registering the pH decrease in an ABL-2 blood gas instrument (Radiometer Copenhagen). The inter-assay CV (n = 5) was found to be 4.1% against a stated CV of 16% in the original method [13].

Results

Figure 2 visualizes the result of fractionation, the bottom dark layer consisting of relatively old rbc. Table 1 compiles the creatine contents and ACh-ase activities in washed samples of both fractions (blood No. 1) as well as those in the fractions of three other blood units. Table 1 shows highly significant differences in both age markers between the two fractions of the four blood units.

Table 1

Typical creatine contents and acetylcholinesterase activities of young and old fractions of four blood units

	Creatine (μg/ml rbc*)		ACh-ase** (ΔpH/h)	
	young	old	young	old
Blood No. 1	31.3	24.2	1.028	0.896
Blood No. 2	38.8	25.7	1.985	1.744
Blood No. 3	39.8	17.7	2.120	1.696
Blood No. 4	35.5	20.1	1.847	1.744

* red blood cells
** acetylcholinesterase (EC 3.1.1.7)

Discussion

All existing rbc separation techniques are based on the fact that rbc density increases with cell age. Routine bloodbank centrifugation does not lead to cell separation [15]. Angle-head centrifugation (to enhance the internal circulation) at 30 °C (to improve the flow properties of rbc) at $39{,}000 \times g$ for 1 hour leads to reasonable cell separation. However, this method requires a special angle-head rotor, is rather cumbersome and can handle only quantities of 80 ml blood [15]. Percoll has been successfully used before, but only on a small scale basis [7, 8]. The proposed method has been shown to separate rbc into a comparatively old and young fraction. The ratio of these fractions can be chosen by carefully assessing the proper Percoll density. Likewise, the 2 fractions can possibly be separated further into subfractions.

The method can be scaled up by simultaneously processing several bags. After removal of the plasma from various blood units, rbc with different antigenic make-up can be mixed with no apparent damage to the cell membranes [16], offering the possibility of preparing hundreds to thousands of ml of relatively young rbc. Scaling up is, however, rather time-consuming and could probably be done much easier with modern blood cell separators [2–4]. Our method seems to be most rewarding for medium scale purposes.

Unfortunately, Pharmacia Sweden cannot guarantee the safe usage of Percoll® for in vivo purposes. Consequently, rbc fractionated with Percoll can only be used for in vitro goals.

The authors wish to thank Messrs. C. A. van Beek and A. van Tiel, department of medical photography Leyenburg-Eye-Hospital, for making the photographs. They are much indebted to Dr. P. Draper, National Institute for Medical Research, London, for most valuable discussions.

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3.3.

3.3. COMPARISON OF THE DEGREE OF HAEMOLYSIS OF YOUNG AND OLD HUMAN RED BLOOD CELLS DURING STORAGE

INTRODUCTION

There is ample evidence that young rbc have better geometric, osmotic and membrane mechanical properties than old rbc (1). In a recent paper we have shown the superior mechanical properties of rbc kept in a high-K, low-Na preservation medium containing mannitol (S 2.3.).

The objectives of this study are

- to compare the degrees of haemolysis (DH's) during storage in young and old rbc-fractions;
- to evaluate whether young fractions would be a better starting material for the preparation of blood control materials.

METHODS AND RESULTS

The fractionation technique has been fully described in S 3.2. The method was shown to use inexpensive means, to be rather simple, not very time-consuming and to yield good age fractionation.

The haemolysis immediately after separation and during storage of both fractions was determined by measurement of Hb in the supernatants of the fractions after centrifugation in Mhct capillary tubes (S 2.3). The intra-assay CV (n=10) at a 0.37 mMol/L Hb-conc was 0.9%. The preserved rbc were shown to be sterile by routine blood cultures. The Table shows highly significant differences between the DH's in both fractions.

Table. Highly significant differences between the degrees of haemolysis in young and old red blood cells

Haemoglobin (Hb) concentrations (mMol/l)

	young rbc			old rbc		
After:	wb *	s **	% ***	wb *	s **	% ***
0 days	8.0	0.01	0.13	8.4	0.015	0.18
24 days	8.0	0.25	3.1	8.4	0.42	5.0
43 days	8.0	1.21	15	8.4	1.66	20

* whole blood

** supernatant

*** ratio of Hb-concentrations in supernatant and whole blood (in %) = degree of haemolysis

DISCUSSION

We did not measure the DH during storage in an unfractionated sample of the particular blood described in the Table. However, we found much lower DH's (about 2% after 43 days of storage) in other unfractionated blood samples (S 2.3: Fig.2). Thus, although there is a statistically highly significant difference between the DH's of both fractions, the separation technique does not yield a better starting material for the preparation of blood control materials.

Since Percoll is claimed by the manufacturer to be impermeable and non-toxic to biological membranes, we tend to ascribe the increased DH of the fractionated rbc to mechanical damage inflicted during the various manipulations (2). More careful attention to the execution of the necessary manipulations

might decrease mechanical trauma (2).

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4.

COMPARISON OF THE RHEOLOGICAL BEHAVIOUR OF HEMOCYTOMETRY CONTROLS AND FRESH PATIENT* EDTA-ANTICOAGULATED BLOOD SPECIMENS

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* To be published in Haematologia.

SUMMARY

For rheological reasons high viscosity specimens are more sensitive to deficiencies, if any, in the aspiration systems of whole blood flow cytometers and consequently are more likely to give erroneous values. For this reason the rheological behaviour of hemocytometry controls was compared with that of patient blood specimens. It is shown that the behaviour of controls and patient specimens of comparable hematocrits is similar at shear rates that are probably occurring during aspiration (exceeding approximately 40 1/s). The hematocrit is shown to be a major determinant of the viscosities of both controls and patient specimens. The results suggest that from a rheological point of view the fundamental IFCC premise is met, "that errors detected by means of control specimens exactly mirror errors occurring with patients' specimens", provided their hematocrits are

taken into account.

KEY WORDS

EDTA-anticoagulated blood; flow hemocytometry control materials; quality control; rheology; viscosity.

INTRODUCTION

Apart from the obvious requirement of stability of values of hemocytometry control materials, Bull (1) states two further desiderata for an ideal control material: "It should be inexpensive and convenient (to encourage frequent use) (2,3) and it should behave like fresh whole blood (so that it can be processed in the same way as blood samples) and thus provide control for the entire analytical process". Likewise, Groner (4) states that for quality control materials "it is desirable that these materials share all relevant characteristics with the specimen (blood sample)".

This requirement implies a set of specific features of each material. It is recognized that the rheological properties are one of these features (5). For a variety of reasons vacuum leaks can occur in the blood aspiration system of Coulter (6) and other counters; this might go undetected if the viscosities of control materials would be much lower than those of patient EDTA-blood specimens. This might result in sluggish and/or incomplete patient blood flow and concomitant erratic values for the patient blood. Our own laboratory experience showed the crucial importance of the viscosity of blood: due to an insidious vacuum leak in the Coulter suction system our "abnormal high" viscosity control (hematocrit (Ht) 63%)

invariably gave too low values. (Ht is the most important single determinant of blood viscosity (7)). Rabinovitch (8) describes that the Coulter quality control material "4C" cannot be used on an Ortho ELT-8 instrument "for the simple reason that its viscosity results in variable aspiration volumes".

A thorough search of the recent literature shows no data whatsoever on the viscosity of blood control materials (1,4,5,9-11).

The objectives of this paper are:

1. to gain preliminary insight in the viscosity characteristics (rheology) of 4 commercial and 4 laboratory-made (3) control materials.
2. to compare these characteristics with those of 4 routine patient EDTA-blood specimens with comparable Ht values.
3. to investigate whether the use of plasma instead of our artificial preservation medium, containing albumin, (2) has any beneficial effect on the rheological behaviour of laboratory-made control materials.

MATERIALS AND METHODS

Viscosity measurement technique

The viscosity measurements in this study were carried out with a Contraves Low Shear 30 viscometer (Contraves AG, CH-8052 Zurich, Switzerland) (7, 12-14), using a cup width of 0.5 mm. The (modified) technique, as described in full detail by Goslinga et al.(14), has previously been shown to be highly reproducible due to automation and rigorous standardization: Coefficients of variation of approximately 3% at the lowest (0.016 1/s) and ca. 0.4% at the highest (118 1/s) shear rates were obtained. In short, salient features include:

1. Refinement of the zero-point setting by applying remote control, thus obviating unsatisfactory vibrations (13,14);
2. Guarantee of accurate measurements by a calibration program with a calibrated standard oil;
3. Consistent treatment of the specimens before the actual measurements by rotating them in a water bath of constant temperature;
4. Invariable start of measurements, after thorough mixing, at the lowest shear rate, proceeding towards higher shear rates to avoid hysteresis variability (7,14);
5. Routine-plotting of viscosity-versus-time curves (not shown) to facilitate the recognition of artefacts such as an air bubble, a small hair, a non-centered bob, clots or fibrin strands in the sample or an inhomogeneous blood sample, thus controlling the quality of the measurement technique;
6. Automatic standardization of the time factor by computer-controlled (HP 9815S) measurements. These measurements are spread over the entire range of shear rates (0.016 to 118 1/s); At every shear rate a specific length of time is required between the beginning of the rotation and the moment of measurement, starting with 110 seconds at a rate of 0.016 1/s and decreasing to 10 seconds at a shear rate of 118 1/s. Thus, the measurements on one sample could be made within five minutes, minimizing the effect of settling out of the red blood cells (rbc) during the measurement (13,14).

To facilitate comparison of the values of the different specimens, the viscosities are graphically represented versus the shear rates in a double logarithmic coordinate system (yielding so-called rheograms: Fig.1, p. 158).

For practical reasons the measurements were generally performed at 37.0 +/- 0.1 ^{o}C, although some samples were also measured at 20.0 +/- 0.1

°C. (Fig.3).

Fresh patient EDTA-anticoagulated blood specimens

Four fresh patient EDTA-anticoagulated blood specimens (Fig. 1: full curves a-d) of varying hematocrits (Ht's) were measured within seven hours after drawing.

Hemocytometry controls

a. Laboratory controls

Two of the measured controls (Fig. 1, curves e and j) were prepared in an artificial (high-K, low-Na) suspending medium, containing 3% albumin, as described before (3,15).

Although this preservation medium is quite satisfactory, there are two reasons to study the possibility of using normal transfusion plasma instead :

1. Further simplification of blood control processing: (compatible) plasma can be stored frozen and consequently is ready at hand;
2. Possibly hemorheological considerations.

In a previous paper (15) we already mentioned that substances such as citrate, mannitol and sucrose are impermeable to rbc and consequently exert an oncotic effect. We found that the citrate concentration in the plasma anticoagulant CPD-A is insufficient to fully counterbalance the impermeable hemoglobin and other osmotically active substances such as lactate (15). This has recently been confirmed by Hoegman et al. (16). We invariably found satisfactory Mean Cell Volume (MCV) constancy after addition of 0.55% mannitol (final concentration) to CPD-A plasma. Nevertheless, our preliminary impression is that neither MCV nor Red cell Distribution Width (RDW) are constant during periods as long as those found in our described control (15). This needs

further investigation. Furthermore, it should be realized that the percentage of mannitol to be added is dependent on the (quite variable) citrate concentrations in the various citrate-containing anticoagulants (17).

One of the objectives of this paper is to study whether the use of plasma instead of the artificial medium has any beneficial effect on the rheological behaviour of laboratory-made control materials (Fig. 1, curves g and k).

b. Commercial controls

One "abnormal high" control viz. Hyland's "Eight-parameter High Abnormal" control, lot No. 3118 H was measured, 30 days before its expiration date (E.D.) (March 1, 1984) (Fig.1, curve f). Its stated Ht was 56.5%. Three "normal" controls were measured: Coulter's "4 C-Plus II Normal" control, lot No. 9378, was measured 7 days before its E.D. (January 9, 1984) (Fig. 1, curve h) (Ht = 36%); Ortho's Hematology control blood, lot No. 83 LO7/746, was measured 4 days before its E.D. (February 5, 1984) (Fig. 1, curve i) (Ht=38%); Hyland's "Eight-parameter Normal" control, lot No. 398N, was measured 33 days before its E.D. (December 31, 1983) (Fig. 1, curve l) (Ht=38%).

RESULTS

Fig. 1 (full curves a-d) shows the well-known non-Newtonian character of fresh patient EDTA-blood specimens (7). The figures accompanying the curves designate the Ht's of the specimens. Although in normal blood Ht is the most important single determinant of blood viscosities (7), inspection of curves a-d shows that other (partly unknown) determinants can decisively affect fresh blood viscosity values over the entire shear rate range.

The dashed curves f, h, i and l in Fig. 1

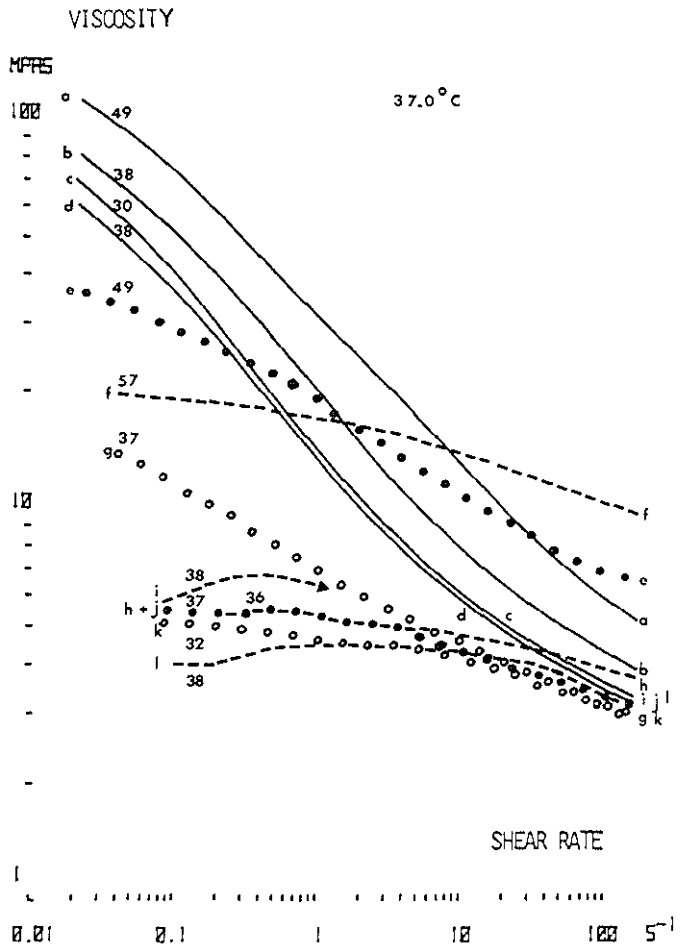

Fig 1. Composite display of rheograms, showing log. viscosities versus log. shear rates of various groups of specimens. All measurements were performed at 37.0° +/- 0.1°C. Figures accompanying the curves designate hematocrits (in %) as measured in a Coulter Counter Model S-Plus II. Full curves (a-d): fresh EDTA-anticoagulated blood specimens; Dashed curves (f,h,i,l): commercial controls, viz.: Hyland's "Eight-parameter Abnormal High" control (f), Coulter's "4C-Plus II Normal" control (h), Ortho's "Normal Hematology" control (i) and Hyland's "Eight-parameter Normal" control (l); Circled curves (e,g,j,k): Laboratory-made controls viz.: open circles (g and k) plasma as the suspending medium and full circles (e and j) artificial suspending medium, containing albumin. For comments see the text.

represent the rheograms of the 4 commercial controls examined. They all show a characteristic (nearly-) Newtonian behaviour. (i.e. independence of viscosities on shear rates). The viscosities of the "Normal" Coulter (h), Ortho (i) and Hyland (l) controls are of the same order of magnitude, and so are their Ht's (36-38%). In contrast, curve f, representing Hyland's "Abnormal High" control, roughly shows three to four times higher viscosities than the "Normal" controls. This clearly demonstrates that (also) in commercial controls Ht's have a decisive influence on their viscosities.

Fig. 1 also shows that the viscosities of the "Normal" controls (h, i, l) approach those of EDTA-blood specimens with comparable Ht's (b-d) only at shear rates exceeding at least 40 1/s (as estimated from the numerical values, which are not shown). On the other hand, Fig. 1 demonstrates that the viscosities of Hyland's "Abnormal High" control (f) at shear rates exceeding 0.5 - 10 1/s are substantially higher than those of fresh EDTA-anticoagulated blood specimens with normal Ht's (Fig. 1, a-d).

The circled curves e, g, j and k in Fig. 1 represent the rheological behaviour of our laboratory-made controls. The full-circled curves (e and j) make up the rheograms of cells in our artificial suspending medium, containing approximately 3% albumin; the open-circled curves (g and k) represent those of cells in mannitol-containing plasma. These media are further to be called artificial and plasma medium, respectively. Controls j (artificial medium) and k (plasma medium) approximately coincide with those of the "Normal" commercial controls h, i and l. Consequently, their viscosities are also much lower than those of comparable EDTA-blood specimens with corresponding Ht's (b-d) (up to shear rates of approximately 40 1/s). At the time of the

measurements specimens j and k were 6 weeks and 11 weeks old, respectively. Specimen j (artificial medium) showed minimal hemolysis, whereas specimen k (plasma) exhibited substantial hemolysis. In contrast, curves e (artificial medium) and g (plasma medium) show a non-Newtonian, more or less parallel, behaviour. However, they fail to coincide with the fresh EDTA-blood curves. Curve e represents the rheogram of the multiparameter hemocytometry control, we recently described in detail (3). The control was 11 weeks old at the time of the measurement, consequently hemolysis was rather extensive (3). Its viscosities at shear rates exceeding 30 1/s are broadly the same as those of the EDTA-specimen with an equal Ht (curve a). Curve g exhibits the rheogram of fresh rbc, suspended in plasma and measured 6 days after preparation.

Fig. 2 shows the viscosity changes of the laboratory-made fresh blood cell-plasma control (corresponding to curve g in Fig. 1) during storage at 4°C as a function of shear rates. At low shear rates the viscosities decrease, whereas at higher shear rates (exceeding 0.1 to 1.0 1/s) viscosities increase. These effects are more pronounced with measurements at 20°C (full curves) than with those at 37°C (dashed curves).

Fig. 3 shows that elevating the measuring temperature from 20°C to 37°C -this range largely spanning worldwide ambient temperatures- results in viscositiy decreases of approximately 30-50% at low (0.3 1/s) and 30-40% at high shear rates (100 1/s) for both a fresh EDTA-blood specimen and for several commercial and laboratory-made controls (see legends to Fig. 3).

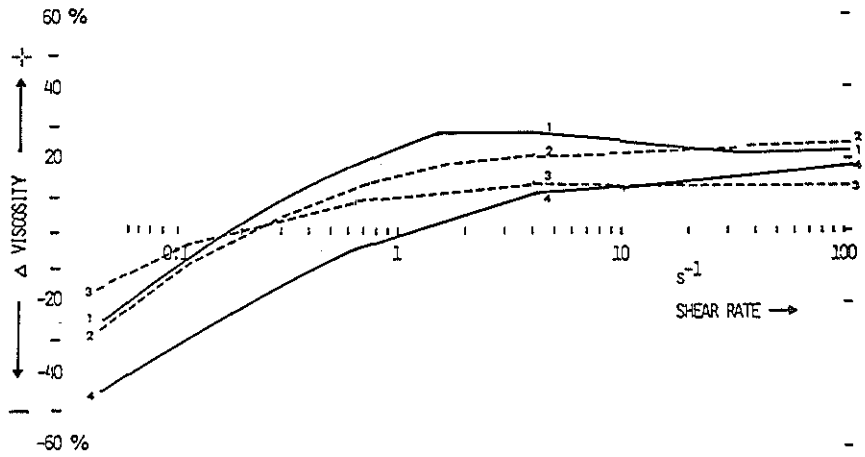

Fig 2. Viscosity changes of the laboratory-made fresh red blood cell-plasma control (corresponding to curve g in Fig. 1) during storage at 4°C as a function of shear rates. The full curves 1 and 4 represent measurements at 20°C, the dashed curves 2 and 3 those at 37°C. Curves 1 and 3 show the changes after 1 month of storage, curves 2 and 4 those after 2 months of storage. At low shear rates viscosities decrease due to decreased aggregation, whereas at higher shear rates (exceeding 0.1 to 1.0 1/s) viscosities increase due to decreased red blood cell deformability. These effects are more pronounced with measurements at 20°C than with those at 37°C.

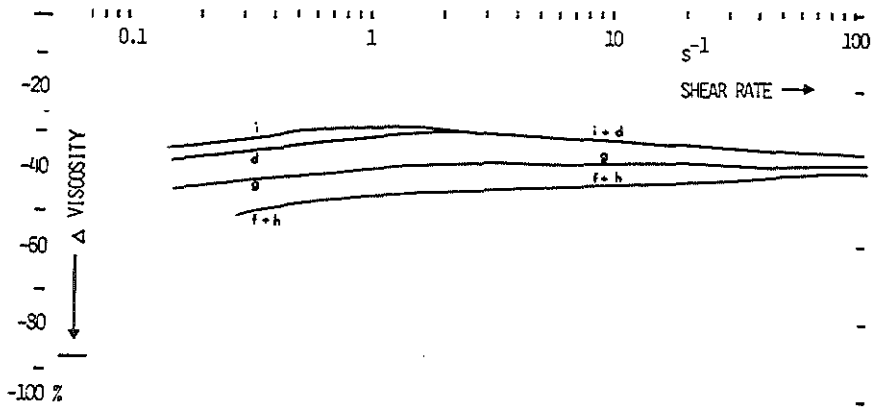

Fig 3. Viscosity decreases due to temperature elevations from 20.0° +/- 0.1°C to 37.0° +/- 0.1°C. The curves represent specimens identical to those of Fig 1 with corresponding letters. Curve d: a fresh EDTA-anticoagulated patient sample; curve i: Ortho's "Normal" control; curve g: laboratory-made fresh red blood cell-plasma control. Curves f and h: Hyland's "Abnormal High" and Coulter's "4C Plus II" controls (coinciding). Viscosity changes vary from 30-50% at low (0.3 1/s) to 30-40% at high shear rates (100 1/s).

DISCUSSION

The fresh patient blood specimens (Fig. 1, full curves a-d) exhibit the well-known non-Newtonian behaviour (7). Viscosities increase exponentially at the lower shear rates due to the presence of the plasma proteins albumin, fibrinogen and immunoglobulins; they overcome the zeta potential between red blood cells (rbc) and aggregate, forming rouleaux, these causing a disproportionate increase in viscosities (7,12,18). It seems appropriate to point out that erythrocyte sedimentation (ES), as routinely determined by the ESR(ate)-test, actually is a low-shear system exerted by gravity (7). At the higher shear rates, rouleaux are dispersed and individual rbc are deformed into ellipsoids with their long axes aligned in the direction of flow (7,12,19).

In contrast, the commercial controls (Fig. 1, dashed curves f,h,i and l) and two of the four laboratory-made controls (Fig. 1, circled curves j and k) show nearly-Newtonian behaviour and their curves approximately coincide (apart from that of specimen f because of its higher Ht). The general rule is that in non-aggregating suspensions much lower viscosities are observed at the lower shear rates, whereas at higher shear rates viscosities (slightly) increase due to decreased rbc-deformability and hence these suspensions tend to behave as (nearly-)Newtonian fluids (20-25). It might be interesting to mention as an example that we found very low ESR-values for Coulter's and Hyland's "Normal" controls (Fig. 1, curves h and l) : 4 mm after 3h and 12-18 mm after 24h of sedimentation. On the other hand strong influences of the plasma composition on ESR-values and consequently on low shear viscosities are well-known from clinical practice.

Finally the remaining two laboratory-made

controls (Fig. 1, circled curves e and g) show non-Newtonian behaviour. However they do not coincide with fresh EDTA-blood specimens with comparable Ht's.

Unfortunately, we can only partly explain the discordant rheological behaviour of our laboratory-made controls. The data show that this discordance is not only due to the nature of the suspending medium : our albumin-containing artificial medium (curves e and j) versus plasma (curves g and k). The non-Newtonian behaviour of the controls e and g at lower shear rates might be related to incomplete discocyte-echinocyte shape transformation and consequently only partly decreased cell-cell interaction.

One of the controls was made from fresh rbc (curve g), the other three controls were 6-11 weeks old and consequently partly hemolyzed. Viscosities are known to change during storage. At corresponding shear rates, hemolyzed packed rbc (packed ghosts) in suspension generally show viscosities slightly lower than those of intact rbc.(23)

Fig. 2 shows the viscosity changes of the fresh plasma control g during storage at 4°C as a function of shear rates. We observed similar typical changes during storage of fresh EDTA-blood : viscosities at (very) low shear rates decrease due to diminished aggregation and viscosities at high shear rates increase due to diminished deformability of the rbc (20-25). Fig. 2 shows that these storage changes are slightly more pronounced when the viscosities are measured at 20°C. However, Fig. 2 also shows that the changes in this particular control specimen are only at most 20% in the probably relevant shear rate range above 40 1/s (see below), which is negligible in comparison to the Ht-effect (vide infra). Similarly, we found negligible viscosity differences (ca. 10% at 20°C) after storage of

the Coulter control. (not shown : it should be mentioned, however, that we measured the control 6 weeks after its expiration date).

Fig. 3 shows that the temperature effect between 20°C and 37°C on the viscosities is similar for both fresh EDTA-blood and the controls, including Hyland's "Abnormal High" control and is only slightly dependent on the shear rate. The viscosity changes amount to approximately 30-50%. It is important to realize that room temperature changes seem to have an equal effect on the viscosities of both patient and control specimens.

Fig. 1 shows similar rheograms for Ortho's control and Coulter's "4C Plus II" control. Assuming that the rheogram of Coulter's control "4C" is similar to that of Coulter's control "4C Plus II", we cannot explain Rabinovitch's statement (8) mentioned in the INTRODUCTION, that Coulter's "4C" control cannot be used on an Ortho ELT-8 instrument "for the simple reason that its viscosity results in variable aspiration volumes".

Figs. 1-3 confirm that the Ht is by far the most important single determinant of the viscosities of both fresh blood and the controls (7) : Fig. 1(f) shows increases in the order of 300-400% for a commercial control and Fig. 1(a) some 200% for fresh EDTA-blood due to Ht-differences, whereas Figs. 2 and 3 show only 20% increase due to deformability decreases and only 30-50% due to temperature changes in the 20°-37°C range. Thus, specimens with high Ht's will be much more sensitive to deficiencies, if any, in the aspiration systems of flow hemocytometers and consequently non-linearity or even erroneous values might occur. This explains our findings of too low values in an "Abnormal high" control, mentioned in the INTRODUCTION. It would be even more important if polycythemia specimens were to be analyzed.

In the Contraves viscometer 28.7

revolutions per minute (rpm) and 98.0 rpm correspond to shear rates of 34.6 1/s and 118.2 1/s, respectively. The fundamental question is what the magnitudes of shear rates are that occur during whole blood flow in the aspiration systems of whole blood flow cytometers. It depends on the vacuum applied and the different geometries in the suction system of the particular instrument used. Consequently, shear rates are likely to markedly vary at various locations in a particular instrument and, a fortiori, in different instruments. However, the appropriate shear rate range is very difficult to estimate; it would require extensive empirical model simulation studies (26). For this reason the viscosities were measured over a wide range of shear rates. Nevertheless, judging from the force with which blood samples are generally sucked up into the hemocytometers, it seems reasonable to speculate that shear rates will be rather high, probably exceeding 40 1/s. As Fig. 1 clearly demonstrates, the magnitudes of occurring shear rates are decisive for the rheological behaviour of fresh EDTA specimens in comparison to that of control specimens : at rates exceeding approximately 40 1/s, their viscosities are numerically comparable. Consequently, the results suggest that from a rheological point of view the fundamental IFCC-premise (27) is met that "errors detected by means of control specimens exactly mirror errors occurring with patients' specimens".

In conclusion, this paper provides insight in the viscosity characteristics of hemocytometry controls in comparison to those of fresh patient EDTA-blood specimens (Fig. 1). Moreover, the article strongly suggests that the use of plasma instead of our artificial suspending medium, containing 3% albumin (3), does not make the rheogram more identical to that of fresh blood and

consequently does not seem to have any beneficial effect on the rheological behaviour of laboratory-made hemocytometry controls.

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Chapter V

SUMMARY AND CONCLUSIONS

1. SUMMARY

In Ch I an INTRODUCTION to AND the OBJECTIVES of this thesis are given. The objectives and the terminology of Internal Quality Control (IQC), External Quality Assessment (EQA) and Quality Assurance (QA) are described in Section 1 (S1). After a short introduction to the significance, the technological progress (S2) and the problems (S3) of haemocytometry (hcy), the objectives of this thesis are described in S4.

The "STATE OF THE ART OF QUALITY ASSURANCE IN HAEMOCYTOMETRY" is reviewed in Ch II. Appropriate QA is a prerequisite for High-Standard Laboratory Performance (HSLP) in the haematology laboratory. Comprehensive QA should comprise the pre-analytical (S2), the analytical (S3) and the post-analytical (S4) phases of laboratory practice.

In S2 the importance of special attention to the pre-analytical phase is emphasized, namely to the requisition of the appropriate tests, to the appropriate preparation of the patient as well as to the appropriate drawing, collection, administration, transport and storage of the blood specimens.

The analytical phase discusses problems of precision and accuracy of the measuring instruments in IQC and EQA programmes (S3).

S 3.1 elaborates on the basic problems of analytical QA in hcy. The concepts of accuracy and precision are elucidated (Fig. 1). Although the advent of flow hcs has dramatically improved precision, accuracy (calibration) is still a major problem. Calibration problems are

compounded by the almost complete lack of reference (ref) preparations due to the instability of blood cells. Difficulties in preparing ref preparations have been discussed. Ref preparations are needed all the more since most routine flow hcs are in fact comparators (cf Ch III, S 3.2). The benificial effect of the only existing ref preparation, that for haemoglobinometry, has been demonstrated. Fig. 2 (p.28) shows the consequences of the absence of ref preparations: substantial differences exist in counting results among different instruments. Flow hcs can achieve quite satisfactory precision, provided their functioning is carefully and continuously monitored in an IQC programme. The preparation of materials intended to contribute to IQC, is described in Ch IV. They serve three basic purposes of IQC: process control, retrospective IQC and blind control, in other words they contribute to the essence of (analytical) IQC viz. continuous self-auditing and continuous attempts at improving performance, as can be monitored by the various precision characteristics. Apart from the general problems, specific problems of calibration and IQC of individual cell parameters are briefly enumerated or referred to. They are either due to abnormalities of the particular blood specimen or to instrument deficiencies or malfunctions.

S 3.2 and Table 1 (p.34) summarize methods primarily aimed at monitoring the analytical precision. Computation of patient values plays an important role in conjunction with the use of control materials. S 3.2 also mentions the approaches to IQC chosen in routine use to deal with the serious inconveniences of lack of ref preparations. Calibration of instruments is approached in essentially three ways:

1. Analysis of a great number of fresh, normal blood specimens by manual or semi-automated

(digital) techniques (cf Ch III).
2. Performance of a few repetitions of carefully performed calibration techniques.
3. Acceptance of stated values of commercial IQC control materials as initial calibration values and comparing the values with those of stated values of other IQC materials and in one or more EQA-schemes. This pragmatic approach is theoretically objectionable, yet is undoubtedly the most widely used. It is a sure sign of the importance of attempts at value assignment techniques of (inter)national bodies, as described in S 3.6.

S 3.3 describes the operational requirements in daily routine if High-Standard Laboratory Performance (HSLP) in the analytical phase is to be met.

In S 3.4 the functions of EQA schemes are mentioned. Fig.3 (p.40) and Table II (p.42) give the order of magnitude of CV's of the different parameters in various schemes. They reflect the state of the art in EQA in hcy, that is commented upon. Finally, some deficiencies of EQA surveys are discussed.

In S 3.5 analytical goals are discussed. In my opinion, the laboratory must provide the most reliable quantitative data possible (as far as costs permit), monitored through objective QA procedures.

In S 3.6 value assignments by (inter)-national bodies are described. They encounter two main problems:
1. The basic technological problems inherent in particle counting and sizing in general (cf Ch III).
2. Specific problems due to the instability of blood cells, necessarily leading to usage of cell substitutes. In haematology the accepted ref count is produced by so-called digital instruments e.g. a Coulter Counter ZBI. The most promising cell substitutes as ref materials are latex particles, that can be

quite accurately sized by light microscopy.(Fig.4 ,p.47)) Two proposed schemes for assigning values to secondary ref materials using fresh blood as an intermedium, are mentioned.(Fig. 5, p.47 and Table III, p.49)

In S 3.7 maintaining the common current practice of initially using IQC materials for calibration purposes is advocated. It is argued that theoretical objections to this practice might well be obviated by application of the recently described BCSH-protocol for precisely assigning values to IQC materials. However, this would require intensive regional or national cooperation.

S 4 emphasizes the importance of the post-analytical phase by quoting La Rochefoucauld's theorem, "it is not enough to have great qualities, one must make good use of them". It is a matter of course that adequate recording, reporting and laboratory-physician communication all contribute to this "good use" of the "great-quality" values hitherto aimed at. Interpretation of values is particularly important in this respect. Consequently several aspects of the interpretation and significance of hcy values are elaborated on in S 4.2, namely the significance of successive counts, ref values and the clinical efficacy of hcy tests are rather extensively discussed.

Finally, aspects of cost-effectiveness and QA are briefly mentioned in S 5. One of the objectives of QA should be to reduce health care costs through appropriate test requisitions.

Ch III discusses the BASIC PRINCIPLES AND PROBLEMS OF HAEMOCYTOMETRY.

After a short introduction in S 1, S 2 deals with manual haemocytometry (hcy).

In S 2.1 it is argued that counting chambers are unlikely to become suitable for calibration

purposes.

S 2.2 enumerates the advantages and disadvantages of the ICSH-recommended Hb determination. The assets of a recently described alternative Hb-method are briefly mentioned.

S 2.3 elaborates on the centrifugal (cf) Hct. In S 2.3.1 the MicroHct (MHct) method is advocated as a potential routine calibration method, in contrast to the official ICSH Macro (ref) method. In S 2.3.2 the cf Hct of normal blood is commented on; it is recommended as an intermedium for value assignments to calibration materials for flow hcy instruments. S 2.3.3 states that the trapped plasma results of the cf Mhct of normal and even of pathological bloods range from 1.18 to 2.25%, provided the Mhct is performed at 13,000 x g for ten min. This is significantly lower than those stated in earlier publications. Discrepancies between cf Hct and flow hcy Hct of pathological bloods are described; they can either be due to departures from normal red blood cell (rbc) deformability or due to hyperosmolar plasma. Finally, it is argued that the diagnostic value of the MCHC, being the ratio of Hb and Hct, is negligible because of its insensitivity to the characteristics of pathological cells. In S 2.3.4 comments are given on the often serious discrepancies between Mhct and flow hcy Hct of artificial blood.

S 3 deals with the principles and problems of flow hcy.

In the Introduction (S 3.1) and in S 3.2 flow hcy instruments are classified into analogue and digital instruments on the one hand and electrical (aperture-impedance or resistive-particle) and optical (light-scattering) instruments on the other hand.

S 3.3 elaborates on the hydrodynamic properties of flow hcy instruments. In S 3.3.1

the flow of fluids in the sensing apertures is discussed. In S 3.3.2 basic fluidic and electronic improvements enabling high resolution cell analyses are described: (2 types of) hydrodynamic focusing, pulse editing and sweep flow are consecutively dealt with.

S 3.4 studies the electrical (aperture-impedance) counting and sizing, as first applied in Coulter Counter instruments. After an introductory (S 3.4.1), aspects of blood cell counting, with special emphasis on counting accuracy, are described in S 3.4.2. Blood cell sizing is dealt with in S 3.4.3. After a primer of sizing theory (S 3.4.3.1), the important shape factor and flow-induced (red) cell shape changes are introduced in the following (sub)sections. They can have an important bearing on the accuracy of rbc sizing (MCV) in pathological blood and consequently on Hct and MCHC (cf S 2.3.3). The overall conclusion is that in impedance-sizing MCV and Hct will be found erroneously more extreme than the "true extreme value" at either extreme side of the mean, whereas the MCHC tends to be erroneously blunted, leading to erroneous constancy. (cf S 2.3.3 and Fig. 6, p. 55). RDW, a quantitative analogue of anisocytosis, can improve the classification of anaemias. However, considerable confusion can be brought about by the various prevailing RDW-definitions, even among the different Coulter Counter Models. Rbc histograms can be used instead to avoid possible confusion. S 3.4.3.5 briefly highlights the major features of plt sizing, such as the influence of anticoagulants and temperature on shape changes and MPV; plt distribution and PDW; inverse, non-linear relationship between MPV and plt count. Finally, a plea is made for strict standardization of Platelet Rich Plasma preparation. S 3.4.3.6 deals with partial wbc differentiation as accomplished in Coulter

counters. Good correlations of Coulter values with those of several other methods are described in the literature. The importance of adequate wbc-threshold setting for accurate wbc counting, sizing and partial differentiation is stressed.

Finally, in S 3.5 some special features of optical (light-scattering) cell counting and sizing are briefly discussed. These can - inter alia - bring about MCV (Hct, MCHC) values at variance with those measured in aperture impedance counters.

Ch IV descibes the PREPARATION AND EVALUATION OF INTERNAL QUALITY CONTROL MATERIALS FOR FLOW HAEMOCYTOMETRY. For a summary of this Ch we refer to its prologue and to the Summaries of the individual Sections.

Ch V (this Ch) consists of the SUMMARY AND CONCLUSIONS.

2. CONCLUSIONS

- Appropriate QA is a prerequisite for High-Standard Laboratory Performance in hcy (Ch II, S 1). The laboratory must provide the most reliable, quantitative data possible, as far as costs permit, monitored through objective QA procedures (Ch II, S 3.5).
- The pre-analytical conditions should be standardized as much as possible (Ch II, S 2).
- The advent of flow hcs has dramatically improved precision, accuracy (calibration) on the other hand is still a major problem due to lack of stable ref preparations (except for Hb) and due to basic technological particle counting and sizing problems (Ch II, Ch III).
- EQA schemes play an important role in assessing the state of the art in QA in

hcy (Ch II, S 3.4).

- Cell (particle) counting is a basic technological problem due to possible particle adhesion, aggregation, coincidence counting etc (Ch III, S 3.4). In practice, digital flow hcs are used to approximate the true counts. (Ch II, S 3.6).
- Accurate (red) cell sizing (MCV, Hct, MCHC determinations) in flow hcy demands simultaneous measurements of individual cell deformability (shape factor)(electrical counters), refractive index (optical counters) and plasma osmolality. This applies especially to pathological rbc. (Ch III, S 3.4.3 and S 3.5). Accurate platelet sizing (MPV, PDW) requires special attention to several factors (Ch III, S 3.4.3.5). (Partial) wbc differentiation (sizing) is successfully accomplished in several multiparameter flow hcs.
- For pragmatic reasons the current practice of (initially) using (commercially-) assigned values of IQC materials for calibration should be maintained. (Supra) regional or national application of a recently described BCSH protocol for assigning values to these IQC materials could obviate objections to the use of IQC materials for calibration purposes (Ch II, S 3.7).
- Mhct of normal, fresh human blood is a practical calibration method for flow hcy Hct, provided it is performed under carefully controlled and specified conditions. On the other hand, there are several possible reasons for discrepancies between flow hcy Hct and cf (M)hct of pathological and/or artificial bloods (Ch III, S 2.3).
- Ch IV describes the methods to prepare IQC

materials in the laboratory. Ease of preparation and low cost provide the means to frequently monitor the instruments, thus contributing to optimization of IQC. Optimization should comprise process control, retrospective IQC and blind control; all these elements contribute to the essence of IQC: continuous self-auditing and continuous attempts at improving laboratory performance. Furthermore, patient MCV, MCH and MCHC can be very useful, additional parameters in IQC, especially if appropriate computer algorithms are applied (Ch II, Table I).

- A plethora of unusual, but important interferences in individual blood specimen hcy can occur. Especially an abnormal MCHC can help recognize them (Ch II, S 3.1.2).
- In flow hcy MCH and MCHC generally give no clinical information additional to the MCV. In contrast, the combination of RDW and MCV enable full classification of anaemias. (Ch II, S 4.2.3; Ch III, S 3.4.3.4).
- Different, prevailing definitions for RDW and PDW (Ch III, S 3.4.3) can lead to considerable confusion.
- One of the objectives of QA should be to reduce health care costs -inter alia- through appropriate test requisitions and interpretations (Ch II, S 4 and S 5).

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References to Chapter IV : See the various subsections of Chapter IV.

SAMENVATTING (Summary in Dutch)

In Hoofdstuk I (H I *) worden de "INLEIDING EN DE DOELSTELLINGEN" van dit proefschrift gegeven. De doelstellingen en de terminologie van Interne Kwaliteits Kontrole (IKK), Externe Kwaliteits Vaststelling (EKV) en Kwaliteits Bewaking (KB) worden beschreven in Sektie 1 (S 1). Na een korte inleiding tot de betekenis, de technologische vooruitgang (S 2) en de problemen (S 3) van de hemocytometrie (letterlijk: het meten van bloedcellen), worden de doelstellingen van dit proefschrift genoemd in S 4.

In H II wordt een overzicht gegeven van de "STAND VAN ZAKEN OP HET GEBIED VAN KWALITEITSBEWAKING IN DE HEMOCYTOMETRIE". Geschikte KB is een eerste vereiste voor Laboratorium-Werk-Van-Hoge-Kwaliteit (LWHK) in het hematologisch laboratorium. Allesomvattende KB dient de pre-analytische (S 2), de analytische (S 3) en de post-analytische (S 4) fasen van de laboratoriumpraktijk te bestrijken.

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* Voornaamste afkortingen (zie ook "Major abbreviations", p. 10; Engelse equivalenten worden, waar nodig, tussen haakjes toegevoegd).

cf	=	centrifugaal
EKV	=	Externe Kwaliteits Vaststelling (≡ EQA)
flow hcs	=	flow hemocytometers
H	=	Hoofdstuk
Hb	=	Hemoglobine
hcie	=	hemocytometrie (≡ hcy)
IKK	=	Interne Kwaliteits Kontrole (≡ IQC)
KB	=	Kwaliteits Bewaking (≡ QA)
LWHK	=	Laboratorium-Werk-van-Hoge-Kwaliteit (≡ HSLP)
MCHC	=	geMiddelde Concentratie van Hemoglobine in rode Cellen
MCV	=	geMiddelde rode Cel Volume
Mhct	=	Microhematokriet
p.	=	pagina
plt	=	bloedplaatjes
rbc	=	rode bloed cellen
ref	=	referentie
S	=	Sektie
Vgl	=	Vergelijk
wbc	=	witte bloed cellen.

In S 2 wordt de nadruk gelegd op het belang van speciale aandacht voor de pre-analytische fase, met name voor het aanvragen van geschikte bepalingen, voor de juiste voorbereiding van de patient, alsmede voor de juiste afname, verzending, administratie, transport en opslag van bloedmonsters.

De analytische fase (S 3) bespreekt de problemen van precisie en juistheid van de meetinstrumenten in IKV en EKV programma's.

S 3.1 gaat uitgebreid in op de fundamentele problemen van analytische KB in de hemocytometrie (hcie). De begrippen "juistheid" en "precisie" worden toegelicht (Fig. 1, p. 24). Ofschoon de komst van de zgn. "flow hemocytometers" (flow hcs; automatische bloedcelmeters) de precisie ingrijpend heeft verbeterd, levert de juistheid (ijking) nog steeds een groot probleem op. IJkproblemen worden verergerd door het vrijwel volledige gebrek aan referentie (ref) preparaten ten gevolge van de instabiliteit van bloedcellen. De moeilijkheden bij de bereiding van ref preparaten worden besproken. Ref preparaten zijn te meer nodig daar de meeste routine flow hcs in feite vergelijkende apparaten zijn (Vgl H III, S 3.2). Het heilzame effekt van het enige bestaande ref preparaat, dat voor de hemoglobine (Hb) meting, wordt aangetoond. Fig. 2 (p. 28) toont de konsekwenties van de afwezigheid van ref preparaten: er bestaan aanmerkelijke verschillen in telresultaten tussen verschillende instrumenten. Flow hcs kunnen een heel bevredigende precisie tot stand brengen, mits een nauwgezet en ononderbroken toezicht op hun werking wordt uitgeoefend in een IKK-programma. In H IV wordt de bereiding beschreven van materialen, die bedoeld zijn om te gebruiken bij de IKK. Zij dragen bij tot de verwezenlijking van drie fundamentele doeleinden van IKK : procesbeheersing, retrospectieve IKK en blinde kontrole, m.a.w. zij dragen bij tot het wezen van de (analytische) IKK nl. ononderbroken zelfkritiek en kontinue pogingen tot het verbeteren van de kwaliteit, zoals voortdurend kan worden nagegaan m.b.v. de verschillende precisie grootheden. Behalve de algemene problemen, worden de specifieke problemen van ijking en IKK van individuele celkenmerken kort genoemd of wordt er naar verwezen. Zij zijn of te wijten aan abnormaliteiten van het specifieke bloedmonster of aan gebreken of slecht funktioneren van de apparatuur.

S 3.2 en Table I (p. 34) vatten de methoden samen, die op de eerste plaats gericht zijn op het voortdurend nagaan van de analytische precisie. Berekening van patientenwaarden speelt daarbij een belangrijke rol in kombinatie met het gebruik van kontrole materialen. S 3.2 vermoemt ook de diverse wijzen van aanpak van IKK, zoals gekozen in de dagelijkse routine, ten einde de ernstige ongemakken t.g.v. gebrek aan ref preparaten zo goed mogelijk het hoofd te bieden. IJking van instrumenten wordt in wezen op drie manieren benaderd :

1. analyse van een groot aantal verse, normale bloedmonsters m.b.v. manuele of semi-automatische (digitale) technieken (Vgl. H III);

2. enkele herhalingen van nauwgezet uitgevoerde manuele ijktechnieken;
3. aanvaarding van opgegeven waarden van kommerciele IKK materialen als initiele ijkwaarden en vergelijking van deze waarden met die van opgegeven waarden van andere IKK materialen en met die in een of meer EKV programma's. Hoewel deze pragmatische benadering theoretisch aanvechtbaar is, wordt zij ongetwijfeld het meest frekwent gebruikt. Het geeft duidelijk het belang aan van pogingen tot ontwikkeling van waardentoekenningstechnieken door (inter)nationale lichamen, zoals beschreven in S 3.6.

S 3.3 beschrijft de werkwijzen die vereist worden in de dagelijkse routine, wil men LWHK in de analytische fase kunnen garanderen.

In S 3.4 worden de funkties van EKV-programma's genoemd. Fig. 3 (p. 40) en Tabel II (p. 42) geven de grootteorde aan van de spreiding c.q. de variatiekoefficienten (CV's) van de verschillende parameters in de diverse programma's. Zij geven de stand van zaken in EKV in de hcie weer, waarop kommentaar wordt gegeven. Tenslotte worden enkele gebreken van EKV programma's besproken.

In S 3.5 worden de analytische doelstellingen besproken. M.i. moet het laboratorium zo betrouwbaar mogelijk (voor zover de kosten dit toelaten) kwantitatieve gegevens verschaffen, gegenereerd via objektieve KB procedures.

In S 3.6 worden de waardentoekenningsprocedures door (inter)nationale lichamen besproken. Zij kampen met twee hoofdproblemen :

1. de fundamentele technologische problemen inherent aan telling en grootte-vaststelling van deeltjes in het algemeen (Vgl. H III);
2. specifieke problemen t.g.v. de instabiliteit van bloedcellen, noodzakelijkerwijs leidend tot het gebruik van celsubstituten.

In de hematologie worden celtellingen, verricht door zgn. digitale instrumenten, b.v. een Coulter Counter ZBI, in het algemeen aanvaard als ref telmethode. Latex deeltjes, waarvan de grootte heel "juist" (accuraat) kan worden vastgesteld m.b.v. lichtmicroscopie (Fig. 4, p. 47), zijn de meest veelbelovende celsubstituten. Er worden twee voorgestelde schema's genoemd voor het toekennen van waarden aan sekundaire ref materialen, waarbij vers bloed als intermedium wordt gebruikt (Fig. 5, p. 47 en Tabel III, p. 49).

In S 3.7 wordt gepleit voor het handhaven van de alledaagse praktijkgewoonte om in eerste instantie IKK materialen te gebruiken voor ijkdoeleinden. Er wordt beargumenteerd dat theoretische bezwaren tegen deze praktijk zeer wel te niet gedaan zouden kunnen worden door toepassing van het onlangs beschreven BCSH-protokol (p. 49) voor het toekennen van waarden aan IKK materialen. Dit zou evenwel intensieve regionale of nationale samenwerking vereisen.

S 4 benadrukt het belang van de postanalytische fase door het citeren van het theorema van La Rochefoucauld, "het is niet voldoende om een goede

kwaliteit te hebben, men moet er ook een goed gebruik van maken". Het spreekt vanzelf, dat adekwate administratie, rapportage en kommunikatie tussen laboratorium en medikus alle bijdragen tot dit "goede gebruik" van de waarden van "goede kwaliteit", waar tot nu toe naar gestreefd werd. Met name de interpretatie van waarden is bijzonder belangrijk in dit opzicht. Daarom wordt in S 4.2 ingegaan op verschillende aspekten van de interpretatie en betekenis van hcie waarden; met name de betekenis van opvolgende tellingen, ref waarden en de klinische efficientie worden vrij uitgebreid besproken.

Tenslotte worden in S 5 in het kort enkele aspekten van kosten en KB genoemd. Een van de doelstellingen van KB behoort te zijn het terugbrengen van kosten in de gezondheidszorg d.m.v. adekwate aanvragen van laboratorium bepalingen.

H III (p. 62 - p. 101) bespreekt de FUNDAMENTELE PRINCIPES EN PROBLEMEN VAN DE HEMOCYTOMETRIE. Na een korte inleiding in S 1, behandelt S 2 de manuele hcie.

In S 2.1 wordt beargumenteerd waarom telkamers waarschijnlijk nooit geschikt worden voor ijkdoeleinden.

S 2.2 somt de voor- en nadelen op van de door de ICSH aanbevolen Hb-bepaling. De voordelen van een onlangs beschreven alternatieve Hb-methode worden in het kort genoemd.

S 2.3 behandelt uitvoerig de centrifugale (cf) Hematocriet (Hct; dit is het volume van de rode bloedcellen als percentage van het totale bloed volume). In S 2.3.1 wordt de MikroHct (Mhct) methode aanbevolen als een potentiele routine ijkmethode, in tegenstelling tot de officiele ICSH-Makro (ref) methode. In S 2.3.2 wordt kommentaar gegeven op de cf Hct van normaal bloed; hij wordt aanbevolen als een intermedium voor waardentoekenning aan ijkmaterialen voor automatische bloedcelmeters. S 2.3.3 beschrijft dat het percentage ingesloten plasma bij de cf Mhct van normale en zelfs van pathologische bloedmonsters varieren van 1.18 tot 2.25 %, mits de Mhct wordt uitgevoerd bij 13.000 x g gedurende tien minuten. Deze percentages zijn signifikant lager dan die opgegeven in vroegere publikaties. Er worden diskrepanties beschreven tussen de cf Hct en de Hct verricht met automatische bloedcelmeters (zgn "flow hcie Hct") bij pathologische bloedmonsters; deze kunnen of te wijten zijn aan afwijkingen van de normale vervormbaarheid van rode bloedcellen (rbc) of aan hyperosmolair plasma. Tenslotte wordt gesteld dat de diagnostische waarde van MCHC (de geMiddelde Hb Concentratie per rode bloedCel, zijnde de verhouding tussen het Hb en de Hct) verwaarloosbaar is vanwege zijn ongevoeligheid voor de kenmerken van pathologische rbc. In S 2.3.4 wordt kommentaar gegeven op de vaak ernstige diskrepanties tussen de Mhct en de "flow hcie" Hct van kunstmatige bloedmonsters.

S 3 behandelt de grondbeginselen en de problemen van de zgn flow hcie

(meting van bloedcellen in een vloeistofstroom, zoals algemeen toegepast bij automatische bloedcelmeters, te noemen flow hemocytometers of flow hcie instrumenten). In de Inleiding (S 3.1) en in S 3.2 worden flow hcie instrumenten geklassificeerd in analoge en digitale instrumenten aan de ene kant en elektrische (apertuur-impedantie) en optische (licht-verstrooiings) instrumenten aan de andere kant. S 3.3 weidt uit over de hydrodynamische eigenschappen van flow hcie instrumenten. In S 3.3.1 wordt de vloeistofstroom in de meetopeningen (aperturen) besproken. In S 3.3.2 worden fundamentele hydrodynamische en elektronische verbeteringen besproken die celanalyses van hoog oplossend vermogen mogelijk maken : (2 typen) hydrodynamische focusering, signaalbewerking ("pulse editing") en de zgn "sweep flow" worden behandeld. S 3.4 bestudeert de elektrische (apertuur-impedantie) hcie, zoals als eerste toegepast in zgn Coulter Counter instrumenten. Na een inleiding (S 3.4.1) worden enkele aspekten van het tellen van bloedcellen, met speciale nadruk op de teljuistheid, beschreven in S 3.4.2. De groottebepaling van bloedcellen wordt behandeld in S 3.4.3. Na een basale inleiding tot de theorie van groottebepalingen (S 3.4.3.1) worden de belangrijke vormfaktor en de door de (vloeistof)stroom teweeggebrachte veranderingen in celvormen beschreven in de volgende (sub)sekties. Zij kunnen een belangrijke invloed hebben op de juistheid van de groottebepaling van rbc (MCV, dit is het geMiddelde rode Cel Volume) in pathologisch bloed en dientengevolge op de Hct en de MCHC (Vgl S 2.3.3). Samenvattend is de konklusie dat bij groottebepalingen m.b.v. impedantie de MCV en de Hct (foutief) extremer gevonden zullen worden dan de "ware extreme waarde" aan beide extreme zijden van het gemiddelde, terwijl de MCHC daarentegen (dientengevolge) foutief "afgestompt" wordt, leidend tot een foutieve konstantheid (Vgl S 2.3.3, p. 68 en Fig. 6, p. 55). RDW (de Rode cel Distributie breedte of Wijdte), een kwantitatief analogon van de anisocytose (d.i. rode cellen van abnormaal-ongelijke grootte) kan de klassifikatie van anemieen (vormen van bloedarmoede) verbeteren. Er kan echter aanmerkelijke verwarring ontstaan door de verschillende, bestaande RDW-definities, zelfs tussen de verschillende modellen Coulter Counters. Rbc-histogrammen kunnen in plaats van de RDW gebruikt worden ten einde eventuele verwarring te vermijden. S 3.4.3.5 legt in het kort de nadruk op de belangrijkste aspekten van de groottebepaling van bloedplaatjes (plt), zoals de invloed van anticoagulantia en de temperatuur op vormveranderingen en de MPV (geMiddelde Plt Volume); plt distributie en PDW (Plt Distributie Wijdte); omgekeerde, niet-lineaire relatie tussen MPV en het aantal plt. Tenslotte wordt een pleidooi gehouden voor strikte standaardisatie van de bereiding van Plaatjes-Rijk-Plasma. S 3.4.3.6 behandelt de gedeeltelijke witte bloedcel (wbc) differentiatie, zoals tot stand gebracht in Coulter Coulters. Er worden in de literatuur goede korrelaties beschreven tussen Coulter waarden en die van verschillende andere methoden. De nadruk wordt gelegd op het belang van adekwate wbc-

drempelinstelling ten einde "juiste" (accurate) wbc tellingen, groottebepalingen en gedeeltelijke differentiatie te kunnen garanderen.

Tenslotte worden in het kort in S 3.5 enkele speciale kenmerken van de optische (licht-verstrooiings) hoie besproken. Deze kunnen o.a. MCV (Hct, MCHC) waarden opleveren, die afwijken van die gemeten in apertuur-impedantie tellers.

H IV beschrijft de BEREIDING EN EVALUATIE VAN INTERNE KWALITEITSKONTROLE MATERIALEN VOOR FLOW HEMOCYTOMETRIE. Uitsluitend bloed, in het algemeen alleen menselijk bloed, is geschikt als potentieel kontrole (en ijk) materiaal voor bloedceltellers. Het is echter wegens zijn instabiliteit slechts ongeveer twee dagen als zodanig geschikt. In dit H worden bereidingsmethoden beschreven om bloed en/of bepaalde bloedkomponenten een aantal maanden geschikt te houden als IKK-materiaal. Het voorkómen van zwelling (MCV-vergroting) en van het stukgaan van rbc (hemolyse) is daarbij een van de grootste opgaven. Het hemolyseprobleem is groter gebleken voor optische dan voor elektrische bloedceltellers, daar de laatste veel minder gevoelig zijn voor hemolyse. Voor een meer gedetailleerde samenvatting van dit H wordt om praktische redenen verwezen naar zijn (Engelstalige) voorwoord (p. 100) en naar de samenvattingen van de individuele (Engelstalige) Sekties.

H V bestaat uit de SAMENVATTING EN KONKLUSIES.

NAWOORD EN DANKBETUIGING
(epilogue and acknowledgements)

De problematiek van de kwaliteitsbewaking van bloedceltellers wordt in ons laboratorium dagelijks in de praktijk gevoeld sinds de komst van de Coulter Counter Model S in september 1972. Een gedeelte van de fundamenten van het hier beschreven onderzoek stamt dan ook al vanaf die tijd. Jarenlang hebben we over een vrij efficient kwaliteitskontrole systeem kunnen beschikken door tegen lage kosten zelf voldoende kontrolemateriaal te bereiden, uitgaande van verlopen transfusiebloed. De storende, technische onvolkomenheden van dit materiaal, alsmede de steeds hogere eisen die de nieuwere generatie celtellers aan kontrole materialen stelden, schreeuwden a.h.w. om vervolmaking daarvan. In maart 1980 werd dan ook hiermee begonnen, ditmaal met de bedoeling de eventuele resultaten in een proefschift neer te leggen. Velen zijn mij daarbij tot onmisbare steun geweest.

Allereerst mijn promotor, Prof. Dr. B.Leijnse, die sindsdien in ontelbare, urenlange besprekingen het onderzoek heeft begeleid en met zijn grote interesse en zijn fundamentele benadering wezenlijke bijdragen heeft geleverd tot de totstandkoming en kwaliteit van dit geschrift. U was voor mij een baken in zee in de moeizame en eenzame strijd tegen de bloedelementen. Het verheugt mij dan ook zeer uiteindelijk vele publikaties met U te hebben mogen verrichten. Uw stiptheid heb ik als aangenaam en uiterst belangrijk ervaren, speciaal voor een promovendus-op-afstand. Dit alles maakte U tot een voor mij ideale promotor, waarvoor ik U zeer erkentelijk blijf.

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De hulp van Jhr.Dr.J.A. van der Does en van de heer J.K.Jagdewsing, resp. direkteur en hoofdanalist van de bloedbank "'s-Gravenhage en omstreken" wil ik in dank memoreren.

Dr.M.S.Harvey (Academisch Ziekenhuis, Leiden), as a tribute to you I prefer to acknowledge you in your mother tongue for your willingness to correct the greater part of my manuscript. This thesis, dealing with quality assurance, equally demands for quality of the vehicle of modern (technological) communication, the English language.

Zonder de gastvrijheid en de bereidwillige en aktieve medewerking van Dr.H.Goslinga, anesthesist, en Ing.J.H.A.Heuvelmans (St.Lucas Ziekenhuis, Amsterdam) was het artikel over het rheologische gedrag van bloedmonsters (p. 152) nooit "geworden". Mijn grote dank voor de publikatie die uit onze samenwerking heeft kunnen ontstaan.

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CURRICULUM VITAE

De schrijver van dit proefschrift is geboren op 12 juni 1938 in Zundert (NB).

Op het St.Norbertuslyceum te Roosendaal (1950-1953) en op het O.L.Vrouwelyceum te Breda (1953-1956) volgde hij het Gymnasium-B.

Hij studeerde Farmacie aan de RijksUniversiteit te Utrecht (Prof. Dr. J.A.C. van Pinxteren) in de jaren 1956-1966 met als hoofdvak farmaceutische en als bijvak biochemische analyse (verricht bij het - toenmalige Philips- Duphar te Weesp). Zijn doktoraalskriptie handelde over "struktuur-werkingsrelaties van coca- en tropa-alkaloiden". In 1967-1968 vervulde hij zijn militaire dienstplicht, waarvan 1 jaar als hoofd van de Apotheek van het voormalig Militair Hospitaal te 's-Gravenhage. In 1968-1971 specialiseerde hij zich in de klinische chemie in het St.Canisius Ziekenhuis te Nijmegen (Drs. H.J.Peters). Sinds eind 1971 is hij werkzaam op het Centraal Klinisch Chemisch Laboratorium van het Leyenburg Ziekenhuis te 's-Gravenhage, alwaar dit proefschrift werd bewerkt.