SPECTROPHOTOMETRY OF HEMOGLOBIN AND HEMOGLOBIN DERIVATIVES

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1. Introduction

Since our first review of the subject appeared in 1965 (58), (spectro)-photometric methods have been increasingly used for the determination of the total hemoglobin concentration in blood (c^*_{Hb}) and for measuring the fractions of the common hemoglobin derivatives (oxyhemoglobin, HbO_2; carboxyhemoglobin, HbCO; hemiglobin or methemoglobin, Hi; sulfhemoglobin, SHb) normally or abnormally—though not infrequently—present in human blood. As for the determination of total hemoglobin, the hemiglobincyanide (cyanmethemoglobin, HiCN) method eventually proved to be superior to the various other methods and came to be increasingly used in clinical chemical laboratories (Fig. 1). After it had been adopted as the preferred method by the Standardizing Committee of the European Society of Haematology (39, 40), it was finally accepted for worldwide use by the International Committee for Standardization in Haematology (ICSH) (15, 16). One of the major reasons for selecting the HiCN method was the suitability of HiCN for the preparation of stable and reliable reference solutions. Prescriptions for the production, distribution, and use of such a reference solution were issued by ICSH together with instructions for carrying out the standardized procedure (15, 16).

Numerous (spectro)photometric methods have been developed for the determination of oxyhemoglobin, either as oxyhemoglobin fraction (c_{HbO_2}/c^*_{Hb}) or as oxygen saturation [$S_{O_2} = c_{HbO_2}/(c_{HbO_2} + c_{Hb})$]. Among these methods are many conventional two-wavelength spectrophotometric methods, most of them involving the use of an isobestic point in the absorption

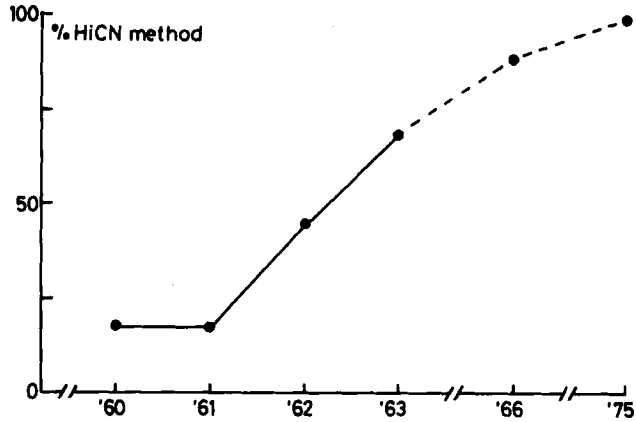

FIG. 1. Relative increase in the use of the HiCN method for the determination of the total hemoglobin concentration in blood, 1960–1975 (55).

spectra of Hb and HbO_2, i.e., a wavelength at which the absorptivity (cf. Section 4.1) of the two components has the same value (58). This yields a single, linear relationship between the oxygen saturation and the ratio of the light absorbances of the sample at the two wavelengths used (65). An important factor in selecting the most suitable pair of wavelengths is avoiding the influence of other absorbing substances in the sample such as bilirubin, lipids, and HbCO (34), or indocyanine green, the commonly used indicator in dye dilution studies (25). Various types of special photometers (oximeters) have been constructed for measuring oxygen saturation *in vitro* as well as *in vivo* (31). In some of these instruments, light reflection is used instead of light transmission (65, 67). The use of reflected light ultimately led to the development of fiber optic oximeters (18, 26, 33), which, though too complicated for routine clinical chemical use, are very suitable for some special applications (77).

Many two-wavelength methods are also used in the determination of the common dyshemoglobins in human blood (HbCO, Hi, SHb). In all of these methods, the samples undergo some kind of pretreatment so that, besides the dyshemoglobin species to be determined, only one other hemoglobin derivative is present. In most of these methods an isobestic point is used. Thus, HbCO can be measured in the system $HbCO/HbO_2$ at $\lambda = 562$ and 540 nm, and in the system HbCO/Hb at $\lambda = 538$ and 578 nm (58). In the former procedure, the blood is gently oxygenated to convert all non-HbCO to HbO_2, while in the latter procedure the sample is deoxygenated using sodium dithionite. A two-wavelength method ($\lambda = 558$ and 523 nm) for the determination of Hi in the system Hi/HbO_2 again involves oxygenation of the sample (58).

Until recently, measuring more than two components in a mixture was much more complicated. The methods involved rather laborious pretreatment of the samples and quite extensive calculations (1, 37, 38, 68). Through the availability of more reliable data as to the absorption spectra of all common hemoglobin derivatives (9, 45) and with the advent of simple and powerful calculators, multicomponent analysis has become much more practicable. It is carried out using conventional spectrophotometers (75) and special instruments (3, 74).

The availability of cheap calculating power led to the tendency of calculating quantities which can be measured directly. An example of this is the calculation of oxygen saturation from oxygen tension. When this is done in addition to the direct measurement, it affords an excellent opportunity for interparametric quality control (54). When it is done instead of the direct measurement, however, it can occasionally be the cause of grave errors (32).

This article deals primarily with spectrophotometric methods that can be carried out with general purpose spectrophotometers. The many special

photometers (hemoglobinometers, oximeters, etc.) presently available for the determination of hemoglobin and hemoglobin derivatives will be mentioned only incidentally.

2. Determination of Total Hemoglobin

This section deals with the determination of the total hemoglobin concentration (c^*_{Hb}) in human blood by means of the standardized HiCN method (15, 16, 57, 71–73). The principles and practice of the method are presented in detail. Also, advice is given as to how the many pitfalls contained in even a strictly standardized procedure can be avoided. The section concludes with a brief description of quality control in hemoglobinometry and a few remarks on methods for the determination of c^*_{Hb} other than the standardized HiCN method.

2.1. Spectral Properties of Hemiglobincyanide

Besides its stability and the relative ease with which other hemoglobin derivatives are converted to it, a reason for selecting HiCN as the preferred compound for hemoglobinometry was its favorable absorption spectrum, with its flat absorption maximum around λ = 540 nm (Fig. 2). The fundamental quantity on which the HiCN method is based is obviously the (milli-

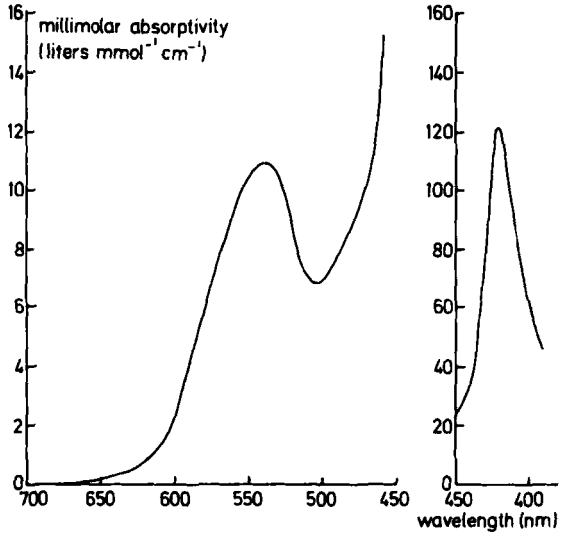

FIG. 2. Absorption spectrum of HiCN. (Based on data from refs. 45 and 58.)

molar) absorptivity at the wavelength of maximum absorption: ϵ^{540}_{HiCN} [cf. Eq. (9) in Section 4.1]. This quantity has been determined by several investigators using different methods. In most investigations, the iron content of the hemoglobin molecule was taken as the basis of the determination of ϵ^{540}_{HiCN} (22, 24, 29, 35, 36, 41, 59, 63, 71). The results thus obtained have been confirmed by the determination of ϵ^{540}_{HiCN} on the basis of both nitrogen (43) and carbon (17). Recently, Hoek *et al.* (14) determined ϵ^{540}_{HiCN} by direct titration of hemiglobin with cyanide. In Table 1, most of the determinations of ϵ^{540}_{HiCN} are summarized. Only the results of Minkowski and Swierczewski (24) and Wootton and Blevin (63) have been omitted. The former have not been included because the standard error of the mean value is not given in the paper, the latter because these results [$\epsilon^{540}_{HiCN} = 10.68 \pm 0.04$ (SEM) ($n = 14$)] are significantly different from all other results taken together. A total mean value for ϵ^{540}_{HiCN} of 10.99 with a total standard error of the mean of 0.01 was calculated for a total of 521 determinations using the equations given in refs. 52 and 73. These data constitute the solid experimental basis on which rests the internationally accepted value for $\epsilon^{540}_{HiCN} = 11.0$.

An essential assumption underlying the determination of the total hemoglobin concentration by absorption photometry—and also the analysis of mixtures of two or more hemoglobin derivatives—is the applicability of Lambert–Beer's law [Eq. (9) in Section 4.1]. The validity of Lambert–Beer's law for HiCN solutions has been repeatedly demonstrated (58, 70). A more severe test has been carried out by Drabkin (11) for HbO_2 solutions. No significant difference in the absorptivity of HbO_2 at $\lambda = 578$, 562, and 542 nm was found when measuring over a concentration range of 0.077 to 38.2 m*M*, with a lightpath length varying from 1.0 to 0.007 cm. Nevertheless, a recent paper (4) has cast some doubt on the general applicability of Lambert–Beer's law to solutions of human hemoglobin. The investigators claim that the absorption spectra of human (oxy)hemoglobin are strongly dependent on the total hemoglobin concentration. Only data pertinent to HbO_2 are given, but it is implied that a similar dependence of the absorption spectrum on the total hemoglobin concentration might be found for HiCN.

Although the experimental evidence in support of this opinion is scanty, the importance of the matter for the whole of hemoglobin spectrophotometry is so great that an experimental reinvestigation seemed unavoidable. Therefore, Zijlstra *et al.* (66) have measured the absorptivity of HbO_2 and HiCN for a wide range of total hemoglobin concentrations. The total hemoglobin concentration in blood was varied from about 0.004 to 10 m*M* in 10 approximately equally distributed steps; the lightpath length varied from 0.007 to 1.000 cm. No concentration-dependent differences in the absorptivity of HbO_2 and HiCN were observed over the wavelength range of 450 to 750 nm. Figure 3 shows that even between the samples with the

TABLE 1

ϵ^{540}_{HiCN} AS REPORTED BY DIFFERENT AUTHORS

Authors	Material	ϵ^{540}_{HiCN} [a]	SEM [b]	n [c]	Method
Meyer-Wilmes and Remmer (22)	Horse Hb	11.0	0.04	12	Fe; *o*-phenanthroline
	Horse Hb	11.0	0.04	12	Fe; $TiCl_3$
Remmer (35)	Human whole blood	11.09	0.03	11	Fe; $TiCl_3$
	Human whole blood	11.19	0.065	4	Fe; complexon
Zijlstra and van Kampen (71)	Human Hb, toluene hemolysis	10.99	0.01	123	Fe; α,α′-dipyridyl
		10.94	0.03	35	Fe; α,α′-dipyridyl
		11.05	0.02	101	Fe; $TiCl_3$
Van Oudheusden *et al.* (59)	Human whole blood	10.99	0.05	10	Fe; α,α′-dipyridyl
	Human whole blood	11.06	0.08	8	Fe; α,α′-dipyridyl
Salvati *et al.* (36)	Human Hb purified on CMC column	10.95	0.03	46	Fe; α,α′-dipyridyl
Morningstar *et al.* (29)	Human whole blood	11.02	0.03	10	Fe; X-ray emission spectrography
	Human washed cells	10.97	0.07	6	
Stigbrand (41)	Human Hb purified on CMC or Sephadex column or by dialysis against Na_2-EDTA	11.00	0.02	55	Fe; sulfosalicylic acid
Tentori *et al.* (43)	Human Hb purified on CMC column	10.90	0.05	55	N analysis
Itano (17)	Human Hb A purified by chromatography	10.88	0.04	16	C analysis
Hoek *et al.* (14)	Human Hb, toluene hemolysis	11.01	0.03	17	Titration of Hi with CN^-

[a] ϵ^{540}_{HiCN}, Millimolar absorptivity (liters $mmol^{-1}$ cm^{-1}) of hemiglobincyanide at $\lambda = 540$ nm.

[b] SEM, Standard error of the mean.

[c] n, Number of determinations.

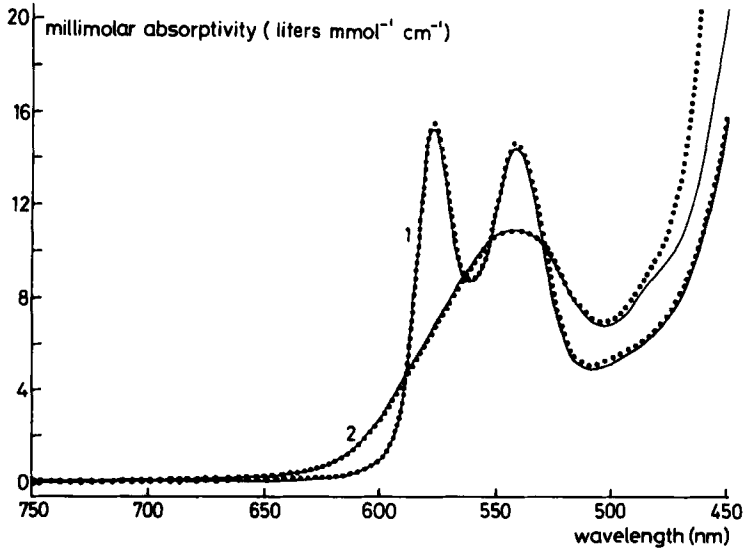

FIG. 3. Millimolar absorptivity of HbO_2 (1) and HiCN (2) as a function of wavelength. Solid lines: l = 0.0071 cm, c_{HbO_2} = 10.609 mM, c_{HiCN} = 10.086 mM. Dotted lines: l = 1.000 cm, c_{HbO_2} = 0.0042 mM, c_{HiCN} = 0.0040 mM. Measurements with a reversed-optics spectrophotometer (HP8450A) for every 2 nm in the wavelength range of 450 to 750 nm. The deviation of the low concentration HiCN spectrum at $\lambda < 500$ nm is due to absorption by the hexacyanoferrate(III) present in the diluent (78). [From Zijlstra *et al.* (66).]

highest and lowest total hemoglobin concentration, no differences in the absorption spectra occur. These results confirm the generally accepted view that, at least in the visible range, Lambert–Beer's law is valid for solutions of hemoglobin and its common derivatives.

2.2. Reagent Solutions

For routine hemoglobinometry, the native blood must be diluted some 200–250 times to lower the hemoglobin concentration to such a level that the absorbance enters a range suitable for measurement with a lightpath length of 1 cm in a general purpose (spectro)photometer. Ideally, the diluent should have such properties that hemolysis occurs immediately, that all hemoglobin derivatives present are rapidly converted into HiCN, and that no turbidity ensues from the presence of plasma proteins and erythrocyte stromata (15). No reagent solution devised up to now strictly fulfills all requirements. This is because these requirements are to some degree mutually incompatible.

Oxidation of the hemoglobin iron, i.e., formation of Hi, is best accom-

plished with the aid of potassium hexacyanoferrate(III) [potassium ferricyanide, $K_3Fe(CN)_6$]. The CN^- ions for the second step of the reaction, the conversion of Hi to HiCN, can be provided by the addition of KCN. Therefore, all reagent solutions contain $K_3Fe(CN)_6$ and KCN, usually at 200 and 50 mg/liter, respectively. Because HCN is a very weak acid (pK_a = 9.1), solutions of $K_3Fe(CN)_6$ and KCN are quite alkaline (pH ≈ 9.6). At this pH, the formation of Hi from Hb or HbO_2 is very slow. The reagent solutions therefore usually contain a third compound to bring the pH so far down that the time necessary for the conversion to Hi becomes reasonably short. Drabkin's solution, the reagent solution in general use before 1961 (71), contains 1.0 g $NaHCO_3$ and has a pH of 8.6. With this reagent solution, the conversion time from Hb or HbO_2 to HiCN is 20 minutes and is mainly determined by the formation of Hi, the addition of CN^- to Hi being very rapid. To attain a further shortening of the conversion time, Chilcote and O'Dea (5) introduced a diluent with pH 7.4 which contains KH_2PO_4 instead of $NaHCO_3$. With this reagent, complete conversion to HiCN takes only a few minutes.

Whereas the requirement of a short conversion time can be achieved to a reasonable degree by lowering the pH of the diluent, the very same change in the composition of the solution favors the appearance of turbidity. This turbidity, which is mainly caused by precipitated plasma protein (primarily γ-globulins, some having an isoelectric point near the pH of the reagent solution) and, to a lesser degree, by erythrocyte stromata, can be effectively prevented by the addition of a small amount of a nonionic detergent such as Sterox SE[1] In the concentration needed, this detergent has no influence on the absorption spectrum of HiCN and it increases the overall reaction velocity by its strong hemolytic action (57).

On the basis of the above considerations, the reagent solution recommended by ICSH (16) has the following composition: 200 mg $K_3Fe(CN)_6$, 50 mg KCN, 140 mg KH_2PO_4, 0.5 ml Sterox SE, and deionized or distilled water to 1000 ml. Sterox SE is an alkylphenol(thiol) polyethylene oxide detergent. It may be replaced by similar detergents such as Nonidet P40,[2] Quolac Nic-218,[3] or Nonic 218.[4] This reagent solution reasonably fulfills the requirements mentioned above. The conversion time is 3 minutes (cf. Fig. 4 in ref. 58). If stored at room temperature in a brown borosilicate glass bottle, the solution keeps for several months. However, there are some imperfections which, in special circumstances, may be the cause of small and sometimes even large errors. Most of these errors can be prevented by quite

[1] Hartmann-Leddon Company, Philadelphia, Pennsylvania.

[2] Shell International Chemical Company, The Hague, The Netherlands.

[3] Unibasic Inc., Arnold, Maryland.

[4] Pennsalt Chemicals Corporation, Philadelphia, Pennsylvania.

simple measures. All errors can be detected if the operator has a good understanding of the procedure. In the following, the possible errors connected with the properties of the reagent solutions are discussed.

The only hemoglobin derivative which is not converted to HiCN is SHb; SHiCN is formed instead. The absorption spectrum of this compound is different from that of HiCN (cf. Figs. 2 and 17). Since ϵ^{540}_{SHiCN} is 8.0, the effect of SHb on the measured value of c^*_{Hb} is slight. It can be calculated that the presence of 1% SHb in the blood causes c^*_{Hb} to be underestimated by only 0.27%. With an SHb fraction of 5% and c^*_{Hb} = 15 g/dl, the measured value of c^*_{Hb} is thus 14.8 g/dl. In practice, the effect may accordingly be considered negligible, as SHb fractions exceeding 5% are seldom encountered.

HbCO is completely converted into HiCN, but the conversion time is considerably longer than for other hemoglobin derivatives (5, 42). As ϵ^{540}_{HbCO} is about 14.3, nonconversion of HbCO to HiCN results in too high values for c^*_{Hb}. The upper limit of this error can be calculated on the basis of the assumption that none of the HbCO present is converted to HiCN during the 3 minutes usually allowed for the reaction (53). For a sample with c^*_{Hb} = 15 g/dl, the maximum error in c^*_{Hb} caused by 10 and 20% HbCO is 3 and 6%, respectively. In practice, the error will be smaller. It can be prevented by increasing the time between the mixing of blood and reagent and the measurement from 3 to 30 minutes. The conversion time can be shortened by increasing the $K_3Fe(CN)_6$ concentration in the reagent solution. However, the number of samples with a HbCO fraction high enough to cause a considerable error in c^*_{Hb} is too small to make worthwhile the use of a special reagent such as the one of Taylor and Miller (42), which contains 1000 mg $K_3Fe(CN)_6$.

In some pathological conditions such as Kahler's disease (multiple myeloma), in which a pathological protein species (paraprotein) is present in the plasma, or in severe infectious diseases in which γ-globulins constitute some 30% of total plasma protein, visible turbidity will develop in the diluted HiCN solution, causing too high values for c^*_{Hb}. In these cases, the addition of one drop of 25% ammonia solution—after the conversion to HiCN has been completed—clears the blood–reagent mixture. In severe lipidemia of the Frederickson V type, chylomicrons may cause turbidity in the diluted HiCN solution. The lipid particles can be removed by diethyl ether extraction and centrifugation. This procedure does not influence the HiCN concentration. That, in the vast majority of cases, no appreciable turbidity develops after mixing blood and reagent solution was shown by the following experiment (46). In 150 consecutive hemoglobin determinations, one drop of ammonia solution was added to the diluted HiCN solution after the absorbance measurement and, thereafter, the absorbance was measured again. In only two cases was there any appreciable drop in the measured value of c^*_{Hb}.

Thus, there is no urgent need for the modified reagent solutions in which no turbidity, or less turbidity, develops when severe plasma abnormalities are present. All proposed modifications narrow down to increasing the ionic strength, which is accomplished either by the addition of NaCl (21, 61) or by increasing the concentration of phosphate buffer (20). The use of a very high NaCl concentration (50 g/liter) in the reagent has been advocated to obtain clear HiCN solutions even in the case of severe leukocytosis (21). However, it seems more convenient to clear the blood–reagent mixture by centrifugation before the absorbance is measured.

The concentration of CN^- in a freshly prepared reagent solution is 0.769 m*M*, which ensures complete conversion of Hi to HiCN (14). However, it has been found repeatedly that the CN^- concentration of a reagent solution decreases as the solution ages (53). CN^- may escape from the solution as HCN, especially when plastic containers are used. When loss of CN^- occurs, the other chemicals in the reagent remaining intact, all hemoglobin in the sample will be converted to Hi, but the subsequent formation of HiCN will be incomplete. As $\epsilon_{Hi}^{540} = 6.8$ (cf. Fig. 14), c_{Hb}^* values up to 40% too low may be found when no HiCN is formed from Hi. The presence of a sufficient amount of CN^- in the reagent solution can be easily checked by repeating the determination of c_{Hb}^* after adding some extra KCN to the diluent. Other methods for this purpose are the use of an ion-selective electrode for CN^- (69) and the Liebig–Denigès argentometric titration of CN^-.

The toxicity of the KCN contained in the reagent solution has been a matter of concern. This has even led to the proposal to substitute a method involving hemiglobinazide (HiN_3) for the hemiglobincyanide method (60). Experience has shown, however, that the risks involved in handling KCN are not too serious. The reagent solution itself is harmless because of the low concentration of KCN. Handling of solid KCN in preparing the reagent solution requires the usual laboratory precautions for toxic substances, which can be easily taken. Moreover, many laboratories use packaged reagents which obviate the handling of solid KCN. As to the proposed HiN_3 method, the problems in substituting HiN_3 for HiCN have been much underestimated (49). Little would be gained by the adoption of this method and many new problems would arise.

The concentration of $K_3Fe(CN)_6$ in the reagent solution is 0.607 m*M*, which is amply sufficient for the oxidation of all hemoglobin iron contained in the diluted blood sample. The amount of $[Fe(CN)_6]^{3-}$ in the reagent solution is stable as long as the solution is not frozen. On freezing and thawing, $[Fe(CN)_6]^{3-}$ is converted to $[Fe(CN)_6]^{4-}$, and no formation of Hi occurs on mixing blood and reagent solution. Consequently, the hemoglobin in the solution will remain mainly in the form of HbO_2. As $\epsilon_{HbO_2}^{540} = 14.3$ (cf. Fig. 12 and Table 5), the measured value for c_{Hb}^* will be about 30% too high. The

occurrence of chemical conversions in $K_3Fe(CN)_6$/KCN reagents on freezing and thawing has been known since the investigations of Michelsen *et al.* (23) and Weatherburn and Logan (62). They reported independently that $K_3Fe(CN)_6$/KCN reagents, when frozen, loose their color and that after thawing, c^*_{Hb} values obtained with these reagents differ from the expected values. Figure 4 shows absorption spectra of a reagent solution before and after freezing and thawing. The absorption spectrum before freezing is identical with that of hexacyanoferrate(III) and the spectrum after thawing with that of hexacyanoferrate(II) [ferrocyanide; $[Fe(CN)_6]^{4-}$].

Zweens *et al.* (78) have demonstrated that the overall reaction occurring in the HiCN reagent on freezing is as follows:

$$2\,[Fe(CN)_6]^{3-} + 2\,CN^- \rightarrow 2\,[Fe(CN)_6]^{4-} + (CN)_2 \qquad (1)$$

Bubbles of $(CN)_2$ can be seen to form on the phase boundary between water and ice. At much higher concentrations of the reactants than present in the HiCN reagent, reaction (1) also proceeds in simple solution at room temperature. However, during freezing, the reaction proceeds at low overall concentrations of the reactants because these become highly concentrated at the moving phase boundary. When ethanol, methanol, ethylene glycol (20 ml/liter), or glycerol (5 ml/liter) is added to the HiCN reagent solution,

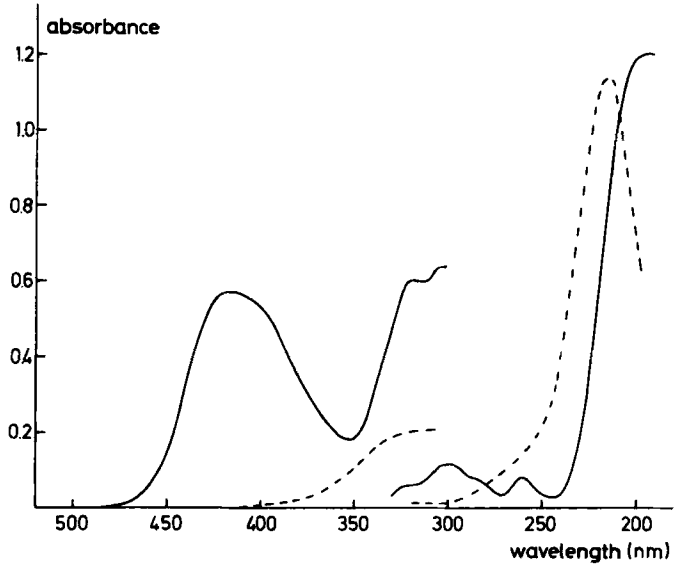

FIG. 4. Absorption spectra of reagent solution before (solid lines) and after (broken lines) freezing and thawing. For the recording from $\lambda = 320$ to $\lambda = 200$ nm, the reagent was diluted 5-fold before, and 10-fold after, freezing and thawing. Optica CF4DR grating spectrophotometer. [From Zweens *et al.* (78).]

decomposition on freezing and thawing does not occur. This is because transition from the liquid to the solid state then takes place abruptly after the solution has become supercooled and a phase boundary does not develop. None of these additions interferes with the formation or the spectral properties of HiCN. Turbidity, if any, is slightly increased by ethylene glycol, but diminished by ethanol and glycerol. Therefore, in circumstances where freezing of the reagent solution is likely to occur, addition of 20 ml/liter ethanol is advisable.

2.3. Handling of Blood Samples

The blood sample may be taken from a freely bleeding capillary puncture (finger; heel, in infants) or from an arterial or venous specimen. The capillary puncture must be deep enough to allow the blood to flow freely. No pressure should be exerted to obtain a sufficient volume of blood from the puncture, for the blood then becomes diluted with tissue fluid. An arterial or venous specimen may be collected into any solid anticoagulant (EDTA, heparin, mixture of ammonium and potassium oxalate). Before sampling from the tube or syringe in which the specimen has been collected, the blood should be well mixed. A tube should be gently tipped end over end 20 times; a syringe should contain a mixing ring or ball.

The optimum dilution of blood in reagent is about 250 times. A dilution of 251 times is achieved by adding 0.02 ml of blood to 5.0 ml of reagent solution. Reagent solution (5.0 ml) is pipetted into a test tube using a bulb-type or graduated-type volumetric pipet with an accuracy of ±0.5%. The accuracy of new pipets should be checked. This may be done by weighing the amount of water the pipet yields when filled to the mark. For larger series of measurements, an automatic pipetting device of the same order of accuracy is advisable.

For the transfer of blood, thick-walled capillary pipets ("Sahli type") can be used. These are one-mark, "to contain" types of pipets. The sample is drawn up to just above the mark and the outside wiped clean with a piece of tissue paper. At the same time, the excess blood is removed from the pipet by gently touching the tip with the tissue paper. The pipet is then placed with the tip at the bottom of the test tube containing the reagent solution and the blood is expelled by blowing gently. The pipet is then partly withdrawn from the solution and rinsed three times with reagent from the upper layer in the test tube. The accuracy of new pipets should be checked. This may be done by weighing the amount of mercury the pipet contains when filled to the mark, by photometry of a dye solution (e.g., patent blue V), diluted using the pipet, or by repeated determination of c^*_{Hb} of a blood sample of known concentration using the pipet. The pipets should be

cleaned daily in detergent, thoroughly rinsed with distilled water, and dried. Cleaning once a week with 0.1 *M* HCl is also advisable.

Instead of the Sahli pipets, disposable capillary pipets can be used. These are glass capillary tubes of uniform bore, made to contain 0.02 ml when completely filled. Other types contain 0.02 ml when filled to a mark. Blood is drawn up and expelled by means of a teat which fits onto the end of the capillary. Care must be taken that no blood from the outside of the capillary gets into the reagent solution. This possible source of error can be eliminated by using break-off capillary tubes. These tubes have a greater length than necessary to contain 0.02 ml, but with a break-off point at the correct length. The capillary is filled from the noncalibrated end. When the calibrated part is filled, it is snapped off at the break-off point and dropped into the test tube with reagent solution and the tube is vigorously shaken.

When, for research purposes, the highest accuracy is to be attained in the determination of c^*_{Hb}, a 200-fold dilution should be made using a 0.5-ml Ostwald pipet and a 100-ml volumetric flask. The volume between the two marks on the Ostwald pipet can be checked by weighing the delivered amount of mercury. The volumetric flask can be checked by weighing after filling it to the mark with distilled water.

2.4. Measurement of the Diluted Hemiglobincyanide Solution

Three minutes after mixing blood and reagent solution, the absorbance of the diluted HiCN solution can be measured. The solution is stable so that the measurement may be postponed for several hours or even days if the solution is stored in a cool and dark space and evaporation is prevented. When a spectrophotometer is used, the wavelength is set at 540 nm and the slit width is adjusted for a half-intensity band width of 1 nm or less. The absorbance is measured using a 1.000-cm cuvette against a similar cuvette filled with water or reagent solution. Both can be used as a blank because the absorbance of the reagent solution at $\lambda = 540$ nm is zero. The total hemoglobin concentration is then calculated using the following equation:

$$c^*_{Hb} = A^{540} f M / \epsilon^{540}_{HiCN} l \qquad (2)$$

where c^*_{Hb} is the total hemoglobin concentration of the blood sample, A^{540} is the absorbance of the diluted HiCN solution at $\lambda = 540$ nm, f is the dilution factor, M is one-quarter of the relative molecular mass of the hemoglobin tetramer: 16114.5 d (2), ϵ^{540}_{HiCN} is the millimolar absorptivity of HiCN at $\lambda = 540$ nm: 11.0 liters $mmol^{-1}$ cm^{-1} (Table 1), and l is the lightpath length: 1.000 cm [cf. Eq. (9) in Section 4.1].

If for f the value 251 is taken, c^*_{Hb} follows from

$$c^*_{Hb}(g/dl) = \frac{A^{540} \times 251 \times 16114.5}{11.0 \times 1.000 \times 10^4} = 36.77 \times A^{540} \quad (3)$$

where 10^4 is the factor for the conversion of milligrams per liter to grams per deciliter.

The wavelength scale of the spectrophotometer can be checked with the help of the mercury emission line at λ = 546.1 nm, the hydrogen emission lines at λ = 656.3 and 486.1 nm, or a filter with light absorption peaks at exactly known positions (e.g., didymium glass). A very simple way to check the wavelength scale is with the aid of a solution of holmium oxide (Ho_2O_3) in perchloric acid. The absorption spectrum of such a solution is shown in Fig. 5. The most suitable peak is at λ = 536 nm, which is quite close to the absorption maximum of HiCN. The absorbance scale is checked using a filter with an exactly known absorbance, such as a carbon yellow filter (Fig. 6). This can also be done with a solution of a compound with exactly known absorptivity and concentration, such as the international HiCN reference solution (Section 2.5).

When a filter photometer is used for measuring the absorbance of the diluted HiCN solution, a filter transmitting a fairly narrow band of light around λ = 540 nm should be employed. The absorbance is measured against water or reagent solution as a blank. The hemoglobin concentration is read from a previously prepared calibration graph or table. The validity of this calibration graph or table should be checked regularly with the aid of HiCN reference solutions. As HiCN solutions strictly obey Lambert–Beer's law, a calibration line (c on abscissa, A on ordinate) can be constructed by simply connecting the coordinates of a single measurement of a reliable HiCN reference solution with the origin (cf. Fig. 5 in ref. 58). If a check on the performance of the photometer is deemed necessary, dilutions of HiCN reference solution with reagent solution are prepared, the concentrations

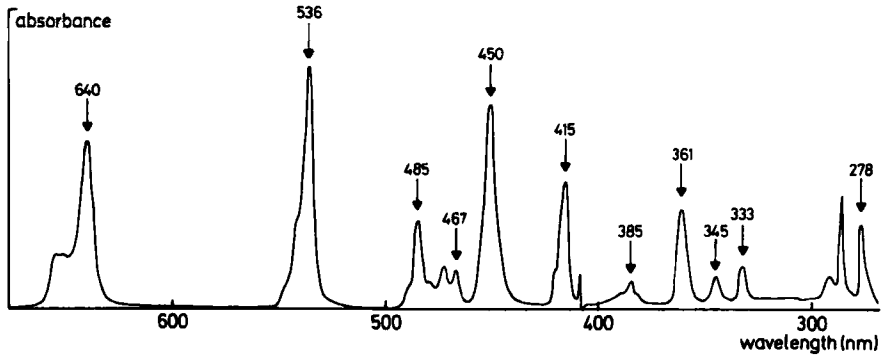

FIG. 5. Absorption spectrum of holmium oxide (Ho_2O_3) in perchloric acid.

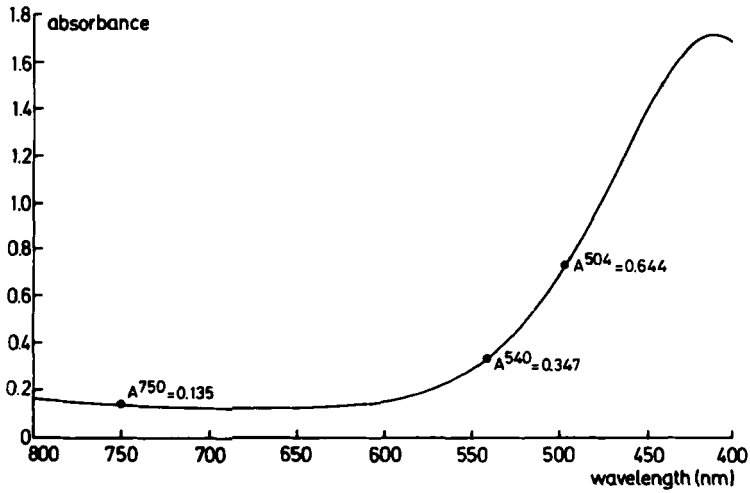

FIG. 6. Absorption spectrum of a carbon yellow filter. The absorbance curve shown is from one of a series of identical filters (Corning HT yellow 1–10) made available by the National Bureau of Standards (Washington, D.C.) to the control laboratories for the international HiCN reference solution (cf. Section 2.5). The absorbance at the three wavelengths used in the control procedure is indicated.

calculated from the diluting factors, and the absorbances measured. The results thus obtained, when plotted on a c vs A graph, should yield a straight line through the origin.

Direct-reading photoelectric hemoglobinometers are special purpose filter photometers with a scale precalibrated in hemoglobin concentration units. The diluted HiCN solution is measured with water or reagent solution as a blank and the hemoglobin concentration read from the scale. The calibration of such instruments should be checked regularly using HiCN reference solutions.

2.5. HEMIGLOBINCYANIDE REFERENCE SOLUTIONS

The international HiCN reference solution is manufactured on behalf of ICSH by the Rijks Instituut voor de Volksgezondheid, Bilthoven, The Netherlands (16), essentially following the procedure described by Zijlstra and van Kampen (72). Briefly, fresh human red cells are washed several times, hemolyzed by toluene, and centrifuged free from debris. All hemiglobin in the sample is converted to HiCN using Drabkin's solution (cf. Section 2.2). This is because the standard procedure for preparing the reference solution was established before the reagent solution containing KH_2PO_4 and nonionic detergent was developed. As the two problems—long

reaction time and turbidity due to plasma protein precipitation—remedied by the modification of Drabkin's solution do not occur in the preparation of reference solutions, it was decided not to change the procedure, the more so because the reagent solution is subject to change (different brands of detergent, addition of ethanol in cold climates, etc.). The international HiCN reference solution is equivalent to a hemoglobin concentration of approximately 600 mg/liter. It is dispensed as a sterile solution (membrane filtration) in sealed 10-ml ampules of amber glass. A batch of this HiCN reference solution has been designated by WHO as International Hemiglobincyanide Reference Preparation (64).

Each batch is periodically tested by a group of laboratories nominated by ICSH (16). In these laboratories, A^{540}, A^{504}, and A^{750} are measured by means of spectrophotometers well calibrated as to wavelength and absorbance scale (cf. Section 2.4 and Fig. 6). The slit width is so chosen that the half-intensity band width is less than 1 nm. Plan-parallel glass cuvettes are used with an inner wall-to-wall distance of 1.000 cm, tolerance 0.5% (0.995–1.005 cm). The measurements are carried out at 20–25°C. The absorbance ratio A^{540}/A^{504} is calculated; it should be between 1.59 and 1.63. The measurement at $\lambda = 750$ nm is a turbidity check. The A^{750} should be less than 0.002 per cm lightpath length when measured using an appropriate blank. In addition to the above absorbance measurements, the absorption spectrum is recorded for $\lambda = 450$–750 nm with a lightpath length of 1.000 cm. Table 2 shows the results of control measurements of a batch of the international HiCN reference solution.

TABLE 2
INTERNATIONAL HEMIGLOBINCYANIDE REFERENCE PREPARATION No. 20600[a]

	A^{750}	A^{540}	A^{504}	$\frac{A^{540}}{A^{504}}$	λA curve
Atlanta	0.000	0.382	0.236^5	1.62	Correct
Cleveland	0.000^5	0.382	0.236^5	1.62	Correct
Groningen (vK)	0.000^5	0.383	0.238	1.61	Correct
Groningen (Z)	0.001	0.380^5	0.236^5	1.61	Correct
Kumamoto	0.000^5	0.384	0.239^5	1.60	Correct
London (Ph)	0.000^5	0.382	0.237^5	1.61	Correct
London (W)	0.000^5	0.382^5	0.238^5	1.60	Correct
Rome	0.000^5	0.382	0.237	1.61	—
Bilthoven	0.000^5	0.381^5	0.237	1.61	Correct

[a] Survey of control (mean values), August 1982. Mean = 0.382 (SD = 0.001, SEM = 0.000^5), corresponding to 560 ± 0.5 mg HiCN per liter (n = 8, Bilthoven not included).

Samples of all batches of the international HiCN reference solution have been stored at 4°C in one of our laboratories and are checked regularly by measuring A^{540} and A^{504}. Results of these long-term stability tests have been reported on several occasions (45, 47, 48). Table 3 shows the results up to the beginning of 1982, covering a period of 18 years. These data demonstrate that the international HiCN reference solution is amazingly stable. There is a very slight tendency for A^{540} to decrease over the years. For the seven batches which have been tested for 10 years or more (Nos. 40400– 10400; 1964–1971), this decrease over the first 10 years of shelf life was 0.5, 0, 1.0, 0.4, 1.5, 1.5, and 1.9%, respectively. The absorbance ratio A^{540}/A^{504} has in all cases been found to be within the range prescribed by ICSH (1.59–1.63), with the exception of No. 40400, after 18 years (1.58), and No. 70400, after 15 years (1.585). Thus, the present extended period of validity for batches of the international HiCN reference solution (3 years) is still very conservative.

The international HiCN reference solution is made available for reference use only to national standards committees for hematological methods or to official government-nominated holders. When there is no committee or official holder, it is distributed to an individual appointed by ICSH. The national holders take care that the international HiCN reference solution is made available to manufacturers and distributors of secondary HiCN reference solutions for the purpose of controlling the quality of their products. Secondary HiCN reference solutions are HiCN solutions made by private or governmental manufacturers, following as closely as possible the procedure used in preparing the international HiCN reference solution. The international HiCN reference solution should always be used in checking the spectral properties of the secondary reference solution.

2.6. Quality Control

After the introduction of the HiCN method in its standardized form (57, 71, 72), the routine use of this method for the determination of c^*_{Hb} steadily increased (Fig. 1). Concomitantly, the precision of the determination of c^*_{Hb} as found in three field trials held in The Netherlands improved considerably (Fig. 7). The spread, expressed as 2 × SD, decreased from 4 g/dl in 1960 to 2 g/dl in 1964 and to 1 g/dl in 1968. The coefficient of variation thus fell from 13 to 3.3% in a period of 8 years.

These data seemed to confirm the original concept of the standardized HiCN method: that the availability of an HiCN reference solution for providing a single calibration point would suffice to yield optimum measuring results. In actual fact, however, this did not prove to be the case. In 1973, 57 laboratories in Europe and Africa participated in an interlaboratory trial organized on behalf of the ICSH Expert Panel on Hemoglobinometry (47).

TABLE 3. LONG-TERM STABILITY TEST OF

Age of batch (years)	Batch no. and year of preparation													
	40400	(1964) $\frac{A^{540}}{A^{504}}$	60400	(1966) $\frac{A^{540}}{A^{504}}$	70400	(1967) $\frac{A^{540}}{A^{504}}$	80400	(1968) $\frac{A^{540}}{A^{504}}$	90400	(1969) $\frac{A^{540}}{A^{504}}$	00400	(1970) $\frac{A^{540}}{A^{504}}$	10400	(1971) $\frac{A^{540}}{A^{504}}$
	A^{540}		A^{540}		A^{540}		A^{540}		A^{540}		A^{540}		A^{540}	
0	0.391	1.61	0.386	1.61	0.405	1.62	0.383	1.60	0.389	1.61	0.395	1.61	0.413	1.61
0.25			0.387	1.61			0.381	1.60	0.389	1.61	0.395	1.61	0.414	1.60
0.5			0.387	1.61	0.404	1.62	0.386	1.61[5]	0.388	1.62	0.396	1.61	0.415	1.61[5]
0.75			0.387	1.61			0.383	1.61						
1	0.389	1.61	0.388	1.61	0.405	1.59[5]			0.388	1.61	0.400	1.62		
1.25					0.403	1.61							0.414	1.61
1.5	0.391	1.61			0.407	1.62	0.386	1.61[5]	0.389	1.62	0.398	1.62	0.414	1.60[5]
1.75					0.405	1.62	0.385	1.61	0.389	1.61	0.397	1.61	0.415	1.61
2	0.391	1.61			0.405	1.62							0.413	1.61
2.25					0.406	1.62			0.390	1.61	0.396	1.62		
2.5	0.390	1.61	0.387	1.61	0.404	1.62	0.386	1.61			0.393	1.60	0.412	1.61
2.75									0.390	1.60[5]				
3	0.391	1.62			0.404	1.61	0.386	1.61			0.393	1.60	0.411	1.61
3.25														
3.5			0.388	1.61					0.387	1.61	0.393	1.61	0.412	1.61
3.75														
4	0.389	1.61	0.387	1.61	0.405	1.61	0.388	1.62	0.387	1.61	0.393	1.61	0.412	1.60
4.5					0.406	1.60[5]								
5	0.393	1.61	0.386	1.61			0.384	1.61	0.387	1.60	0.393	1.60	0.411	1.61
5.5					0.404	1.62								
6	0.390	1.62					0.382	1.60	0.386	1.60	0.392	1.60	0.407[5]	1.61
7					0.402	1.61	0.383	1.60	0.386	1.61	0.390	1.60		
8	0.394	1.61	0.386	1.61	0.405	1.60	0.383	1.61	0.383[5]	1.60[5]				
9	0.390	1.60			0.404	1.61	0.381	1.60						
10	0.389	1.60	0.386	1.61	0.401	1.61					0.389	1.59	0.405	1.61
11	0.390	1.60	0.381[5]	1.60					0.378	1.62			0.405[5]	1.61
12	0.389	1.59					0.382	1.60			0.387[5]	1.60		
13	0.386	1.59[5]			0.400	1.61			0.382[5]	1.61[5]				
14			0.381	1.60			0.386[5]	1.60[4]						
15					0.405	1.58[5]								
16	0.387[5]	1.59	0.380	1.60										
17														
18	0.391[5]	1.58												

The material to be tested consisted of two fresh blood samples (containing EDTA as anticoagulant), two glycerol-containing hemolysates, and an HiCN reference solution. The "true" values were derived from the results obtained by members of the Expert Panel on Hemoglobinometry. Figure 8 shows that the spread in the values obtained for the blood samples and the hemolysates is considerably greater than that in the values obtained for the HiCN reference solution. A similar result was obtained in another interlaboratory trial

INTERNATIONAL REFERENCE SOLUTIONS

Batch no. and year of preparation															
20400	(1972) $\frac{A^{540}}{A^{504}}$	30400	(1973) $\frac{A^{540}}{A^{504}}$	40500	(1974) $\frac{A^{540}}{A^{504}}$	50500	(1975) $\frac{A^{540}}{A^{504}}$	60500	(1976) $\frac{A^{540}}{A^{504}}$	70500	(1977) $\frac{A^{540}}{A^{504}}$	80500	(1978) $\frac{A^{540}}{A^{504}}$	90500	(1979) $\frac{A^{540}}{A^{504}}$
A^{540}		A^{540}		A^{540}		A^{540}		A^{540}		A^{540}		A^{540}		A^{540}	
0.408	1.61	0.403	1.61	0.382[5]	1.60	0.379	1.61	0.392[5]	1.61	0.394[5]	1.60	0.396	1.60	0.392[5]	1.61
0.408	1.61	0.402	1.61	0.380	1.61	0.381	1.61	0.394	1.61	0.396	1.61	0.393	1.61	0.392	1.61
0.408	1.61	0.403	1.62	0.381	1.60	0.381[5]	1.61	0.392	1.61	0.395	1.61	0.394	1.60	0.393[5]	1.61
														0.392[5]	1.61
0.408	1.62	0.402	1.61	0.378	1.61	0.379[5]	1.60	0.393[5]	1.60	0.395	1.61	0.393[5]	1.61	0.392[5]	1.60
		0.401	1.61												
		0.403	1.60	0.379[5]	1.62	0.381	1.60	0.393[5]	1.61	0.394	1.60[5]	0.395	1.61	0.392[5]	1.61
0.406	1.61			0.380[5]	1.61	0.382	1.60	0.392	1.61	0.394	1.61	0.393[5]	1.61	0.393	1.60
		0.401	1.61												
						0.380	1.60	0.391	1.61			0.393	1.61	0.391[5]	1.60
0.404	1.61	0.400	1.61							0.392	1.61	0.393	1.61		
												0.393[5]	1.61	0.388	1.61
0.407	1.60	0.403[5]	1.60[5]											0.390[5]	1.60
								0.391	1.62			0.392[5]	1.60[5]		
0.405	1.61	0.399	1.61												
0.402	1.60[5]					0.379[5]	1.61			0.391[5]	1.61				
								0.387[5]	1.61[5]						
		0.400[5]	1.60			0.376[5]	1.62								
0.399	1.61			0.376	1.61										
		0.396	1.62												

(55) in which, besides an HiCN reference solution, two hemolysates also had to be measured (Fig. 9). It can be concluded from the distribution of the crosses in Fig. 9 that the errors made in measuring the HiCN reference solution are random errors, whereas the distribution of the dots show that, in the results obtained for the hemolysates, systematic errors play a considerable role (cf. Section 6).

Since the results obtained for the HiCN reference solutions are reasonably good (coefficient of variation $< 1\%$), it is improbable that photometric errors have caused the unsatisfactory results for the hemolysates and the whole blood samples. Also, the absence of any significant difference between these two kinds of samples (Fig. 8) excludes nonhomogeneity of the blood samples, a possible result of insufficient mixing before the dilution with reagent solution is made, as an important source of error in the determination of c^*_{Hb} in the whole blood samples. Thus, as causes of error, there remain incomplete conversion to HiCN, resulting from the use of faulty reagent solutions (cf. Section 2.2), and incorrect dilution, due to the use of non- or falsely cali-

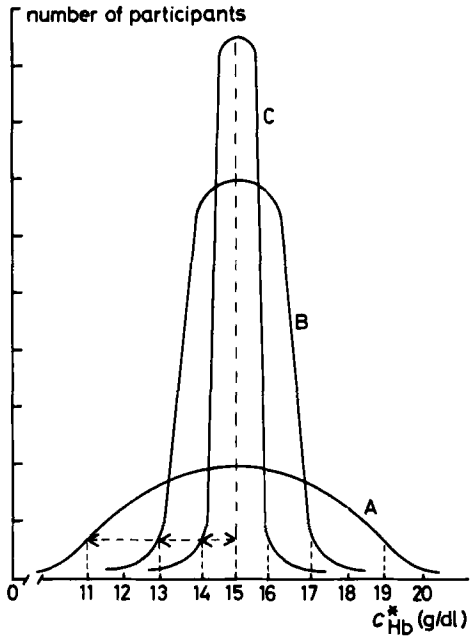

FIG. 7. Results of three field trials concerning the determination of c^*_{Hb} in Dutch hospitals. All data have been recalculated to a mean value of 15 g/dl. The horizontal arrows indicate 2 × SD. A = 1960; B = 1964; C = 1968.

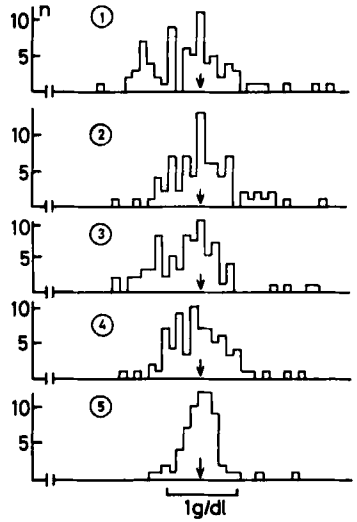

FIG. 8. Histograms of the results of determinations of c^*_{Hb} in an international trial at 67 laboratories. 1 and 2, whole blood; 3 and 4, hemolysates; 5, HiCN reference solution (A^{540} converted into c^*_{Hb} of a hypothetical blood sample). The arrows indicate the values obtained by the reference laboratories.

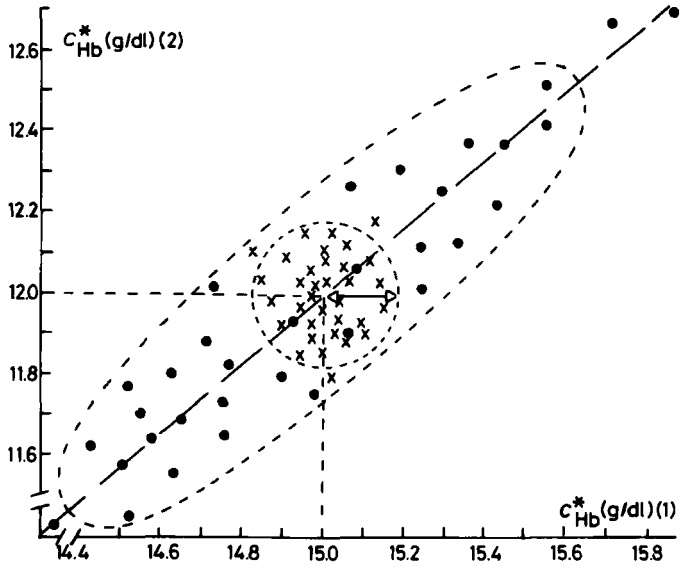

FIG. 9. Youden plot of the results of an interlaboratory trial. The crosses and dots are the result of measurements of HiCN reference solution and hemolysates, respectively (cf. Appendix).

brated pipets and/or incorrectly adjusted dilutors. It is evident from these trials that these two sources of error may play an important part in the daily practice of measuring c^*_{Hb} by the standardized HiCN method.

For the detection of conversion and dilution errors in routine hemoglobinometry, some kind of blood-like preparation with known c^*_{Hb} is necessary. Reasonably stable concentrated hemoglobin solutions of known c^*_{Hb}, prepared by means of toluene hemolysis of human blood and sterilized by membrane filtration, are now commercially available for intralaboratory quality control (47). With the aid of an HiCN reference solution to calibrate the measuring instrument and a concentrated hemoglobin solution to check the HiCN method as a whole, it should be possible for every clinical chemical laboratory to ensure that valid results are obtained.

2.7. Other Methods for the Determination of Total Hemoglobin

Although it is generally recognized that the standardized HiCN method is the preferred method for the determination of c^*_{Hb} in all circumstances, some other procedures are also in use for routine hemoglobinometry. The reasons for using these other methods are based on considerations from

outside the methodology of hemoglobinometry proper. In constructing automated analyzers, it has been considered more practical to use an HiCN method in which the absorbance is measured at a constant, short time after mixing blood and reagent solution, instead of waiting for complete conversion of all hemoglobin to HiCN. In other constructions, the use of HbO_2 instead of HiCN has been chosen for reasons of speed of operation and simplicity of design.

Another reason for using a procedure different from the standardized HiCN method has been the incorporation of a determination of c^*_{Hb} in an oximeter employing a two-wavelength method with an isobestic point (cf. Section 4.2). The absorbance measured at the isobestic wavelength is then linearly related to the total hemoglobin concentration and can easily be used for a readout of c^*_{Hb}. When, by multicomponent analysis, the concentration of all the hemoglobin derivatives in a blood sample is determined (cf. Section 4.5), the sum total of these concentrations is, of course, equal to c^*_{Hb}.

When other methods for the determination of c^*_{Hb} are used, these should be compared regularly with the standardized HiCN method. To this end, some blood samples should be measured simultaneously using the two methods. It is not a good practice to check the other method only with the concentrated hemoglobin solutions mentioned in Section 2.6.

3. Absorption Spectra and Millimolar Absorptivities of Hemoglobin and Hemoglobin Derivatives

In this section, spectral absorbance data are provided for hemoglobin and its common derivatives. Most of the data have been published previously (9, 45, 58), but some new material has been added. The importance of reliable absorption spectra and accurate values for the absorptivities of hemoglobin and its common derivatives has considerably increased since multicomponent analysis of mixtures of hemoglobin derivatives has become feasible in the clinical chemical laboratory (3, 74–76).

3.1. Sample Preparation and Measurement Procedure

Apart from the special procedures necessary to prepare the various hemoglobin derivatives, two slightly different methods were used in the preparation of the hemoglobin solutions. In most cases, the procedure as described in ref. 58 and, in more detail, in ref. 45 was used (procedure 1). This leads to quite clear hemoglobin solutions. In a later series of experiments, the solutions were prepared simply by hemolyzing the blood in a syringe with the

help of a small amount of Sterox SE and pressing the hemolysate without air contact through a cotton-wool filter (75) (procedure 2). This yields hemoglobin solutions which are not as clear as those provided by the first procedure, but the absorption data obtained are very similar.

All specimens were obtained from healthy, nonsmoking humans and provided with heparin as anticoagulant. In the first procedure, the erythrocytes were washed three times with an isotonic saline solution. After the third washing, the packed cells were brought to 1.25 times the original blood volume by adding 0.7% Sterox SE solution and the hemolysate was filtered through a folded paper filter after centrifugation for 10 minutes. Finally, c^*_{Hb} was adjusted to about 8 g/dl. In order to obtain deoxygenated hemoglobin (Hb), sodium dithionite was added to the hemolysate to a molar ratio $Hb/Na_2S_2O_4$ of 1/5. To obtain oxyhemoglobin (HbO_2), the hemolysate was oxygenated in a revolving glass tonometer flushed with pure O_2. To obtain carboxyhemoglobin (HbCO), the hemolysate was saturated with carbon monoxide in a revolving glass tonometer flushed with pure CO. To obtain hemiglobin (methemoglobin; Hi), finely powdered $K_3Fe(CN)_6$ was added to the hemolysate in two steps to a molar ratio of $Hb/K_3Fe(CN)_6$ of 1/3. The pH of the resulting Hi solution was checked using a glass electrode; only solutions with pH 7.0–7.4 were accepted for the absorbance measurements. To obtain hemiglobinnitrite ($HiNO_2$), $NaNO_2$ was added to the hemolysate to a molar ratio $Hb/NaNO_2$ of 1/6 (50, 51). To obtain hemiglobinazide (HiN_3), NaN_3 was added to an Hi solution prepared as previously described to a molar ratio Hi/NaN_3 of 1/6. For the absorbance measurements at $\lambda <$ 450 nm, all solutions were diluted with water either 10 times (Hb, HbO_2, HbCO) or 20 times (Hi, $HiNO_2$, HiN_3). Small amounts of $NaNO_2$ and NaN_3 were then added to the diluted $HiNO_2$ and HiN_3 solutions, respectively.

When the second procedure, with cotton wool filtration as the only clearing procedure (75), was used, tonometry with O_2 and CO for preparing the HbO_2 and HbCO solutions was carried out with fresh whole blood. Hb-containing samples were prepared by tonometry of whole blood with a gas mixture containing 5% CO_2 and 95% N_2. No $Na_2S_2O_4$ was used.

Sulfhemoglobin-containing samples were prepared according to Siggaard-Andersen *et al.* (9, 37). Fresh whole blood was oxygenated by tonometry with pure O_2 and then centrifuged. The packed cells were then incubated for 30 minutes with an equal volume of a freshly prepared solution containing 40 m*M* Na_2S and 75 m*M* HCl (pH 7.5). Finally, excess H_2S was removed by tonometry with O_2. By this procedure, a blood sample is obtained containing 15–25% SHb. Sulfhemiglobin (SHi) was obtained by adding $K_3Fe(CN)_6$ to an SHb-containing solution after ultrasonic lysis of the erythrocytes; sulfhemiglobincyanide (SHiCN) was prepared by the addition of $K_3Fe(CN)_6$ and KCN.

Spectral absorbance curves of the hemoglobin solutions obtained according to the first procedure were made by means of an Optica CF4DR recording spectrophotometer. The absorbance in the regions of maximum absorption was also measured manually on an Optica CF4. Both instruments were calibrated for wavelength using mercury emission lines and for absorbance using a carbon yellow filter (cf. Section 2.4). Both instruments had been checked to ensure the absence of stray light and tested as to photometric linearity. The scale of the Optica CF4DR recorder is electronically linearized for absorbances up to 1.000. Absorption spectra of the hemoglobin solutions prepared by means of the second procedure were obtained with an HP8450A reversed-optics spectrophotometer with built-in facilities for processing and storing the absorbance data. The instrument was checked by means of a carbon yellow filter. The absorption spectra of the three sulfhemoglobin derivatives were recorded using an Aminco-Chance split-beam spectrophotometer with the reference cuvette filled with the same solution as the sample cuvette, but without the SHb derivative. The absorption spectrum of SHb was also obtained with the aid of the HP8450A, using the computing facilities of this spectrophotometer for making the necessary corrections for the absorbance of the other hemoglobin derivative (HbO_2) in the sample.

In each case, concentration and lightpath length were chosen in such a manner that the absorbances were, as far as possible, in the range of near-maximum photometric accuracy (cf. Fig. 2 in ref. 58). The smaller layer thicknesses (l = 0.013 and 0.005 cm) were attained by using 0.100-cm plan-parallel glass cuvettes with 0.087- and 0.095-cm glass inserts (Hellma Benelux, The Hague, The Netherlands) (cf. Fig. 13 in ref. 58 or Fig. 2 in ref. 75). The lightpath length of all cuvettes was determined by comparative measurements of various dilutions of a concentrated HiCN solution, taking a certified 1.000-cm plan-parallel glass cuvette as a primary standard (45, 58).

The calculation of the millimolar absorptivities and the calibration of the ordinate of the spectral absorbance curves in absorptivity units require that the concentration of the compound measured be exactly known. In order to obtain the exact concentration values, all samples, except for the SHb derivatives, were diluted with HiCN reagent solution (cf. Section 2.2) and the absorbance measured at λ = 540 nm in a certified 1.000-cm cuvette. The concentration of hemoglobin derivative X in the original sample was then calculated using the equation

$$c_X \text{ (mM)} = A^{540}_{\text{HiCN}} f / \epsilon^{540}_{\text{HiCN}} l \tag{4}$$

where f is the dilution factor, l = 1.000 cm, and $\epsilon^{540}_{\text{HiCN}}$ = 11.0 liters mmol^{-1} cm^{-1}. The millimolar absorptivity of hemoglobin derivative X at wavelength λ followed from

$$\epsilon^{\lambda} \text{ (liters mmol}^{-1} \text{ cm}^{-1}) = A^{\lambda}/c_X l \quad (5)$$

where A^{λ} is the absorbance measured at wavelength λ for the original X-containing sample and l the lightpath length used in this measurement.

For the SHb derivatives, a special procedure had to be used, first, because these compounds must always be measured in the presence of other hemoglobin derivatives (pure solutions cannot be prepared) and, second, because they cannot be converted to HiCN. This procedure is described in Section 3.8.

Figure 10 shows the visible-range absorption spectra of the most important hemoglobin derivatives. The samples were prepared by the second procedure, except for HiCN, for which a reference solution was used (cf. Section 2.4). The measurements were carried out with the HP8450A reversed-optics spectrophotometer. The spectral absorbance curve of Hb is based on measurements of the blood of 10 donors, and that of HbO_2, HbCO, and Hi is based on the blood of 14, 8, and 16 donors, respectively. The absorption spectrum of SHb is based on a single blood sample.

3.2. Deoxygenated Hemoglobin (Hb)

The absorption spectrum of Hb is shown in Fig. 11 and, for the visible range, also in Fig. 10. The sample preparation for the data of Fig. 11 was according to procedure 1 as described in Section 3.1. In Table 4, the milli-

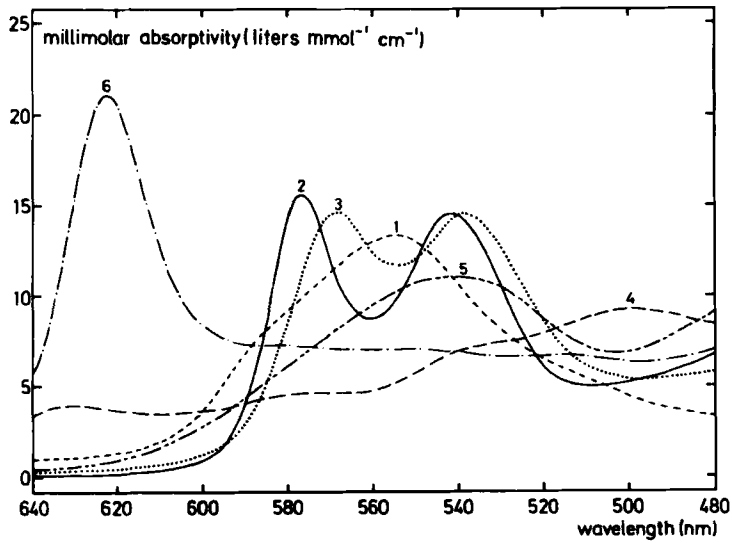

Fig. 10. Absorption spectra of (1) Hb, (2) HbO_2, (3) HbCO, (4) Hi, (5) HiCN, and (6) SHb.

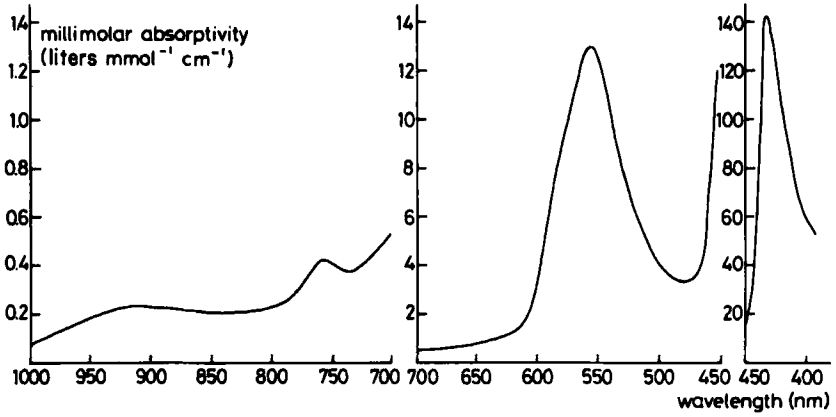

FIG. 11. Absorption spectrum of Hb. (Based on data from refs. 45 and 58.)

molar absorptivity of Hb is given for the maxima and minima in the spectral absorbance curve and for some crossover wavelengths with the spectra of various hemoglobin derivatives (isobestic points). Part of the isobestic points can also be seen in Fig. 10. The isobestic point with HbO_2 in the near infrared is found at $\lambda = 815$ nm. In the spectrophotometric determination of the oxygen saturation described in Section 4.2, an isobestic wavelength of 800 nm is used. This is the value which is consistently found when measure-

TABLE 4
MILLIMOLAR ABSORPTIVITY OF Hb

λ (nm)	ϵ^{λ} (liters mmol^{-1} cm^{-1})	Comments
910	0.23	Maximum
850	0.20	Minimum
815	0.22	Isobestic with HbO_2
760	0.43	Maximum
736	0.37	Minimum
600	3.20	Isobestic with Hi
586	7.23	Isobestic with HbO_2
579	8.86	Isobestic with HbCO
569	11.27	Isobestic with HbO_2
561.5	12.54	Isobestic with HbCO
555	13.04	Maximum
548.5	12.46	Isobestic with HbO_2
547.5	12.37	Isobestic with HbCO
528.5	7.71	Isobestic with Hi
522	6.42	Isobestic with HbO_2
506.5	4.81	Isobestic with HbO_2
478	3.31	Minimum
431	140.0	Maximum

ments are made on samples which have been deoxygenated by tonometry with a CO_2/N_2 mixture. When $Na_2S_2O_4$ is used for deoxygenation, a somewhat longer isobestic wavelength is found for Hb/HbO_2. The difference looks considerable, but has little practical importance because the spectral absorbance curves of Hb and HbO_2 are very flat in this region. Table 4 and Fig. 10 show that $\lambda = 548$ nm is almost an isobestic triple point for Hb, HbO_2, and HbCO. Using procedure 2 and the HP8450A spectrophotometer, a value of 13.30 liters $mmol^{-1}$ cm^{-1} was found for the millimolar absorptivity in the absorption maximum at $\lambda = 555$ nm.

3.3. Oxyhemoglobin (HbO_2)

The absorption spectrum of HbO_2 is shown in Fig. 12 and, for the visible range, also in Figs. 3 and 10. The sample preparation for the data of Fig. 12 was according to procedure 1. In Table 5, the millimolar absorptivity of HbO_2 is given for the maxima and minima in the spectral absorbance curve and for some isobestic points (cf. Fig. 10). Some comment on the position of the isobestic point for Hb/HbO_2 in the near infrared is given in Section 3.2. Using procedure 2 and the HP8450A spectrophotometer, a millimolar absorptivity of 15.42 and 14.61 liters $mmol^{-1}$ cm^{-1} was found in the maxima at $\lambda = 577$ and 542 nm and of 8.71 and 4.96 liters $mmol^{-1}$ cm^{-1} in the minima at $\lambda = 560$ and 510 nm, respectively.

3.4. Carboxyhemoglobin (HbCO)

The absorption spectrum of HbCO is shown in Fig. 13 and, for the visible range, also in Fig. 10. The sample preparation for the data of Fig. 13 was

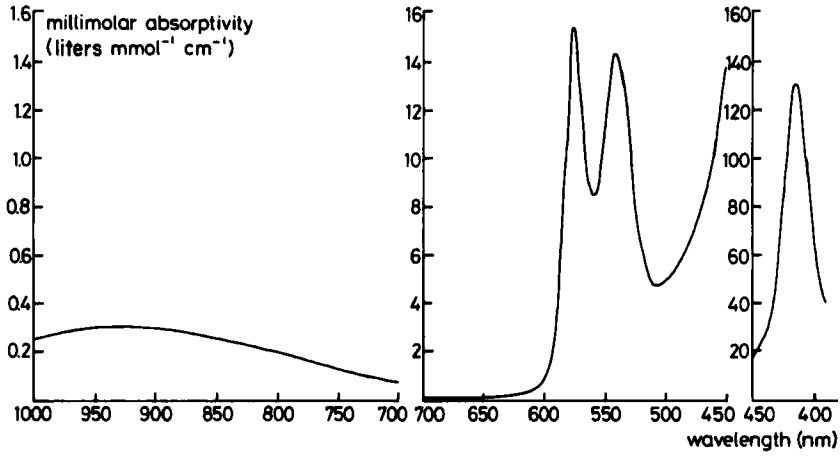

FIG. 12. Absorption spectrum of HbO_2. (Based on data from refs. 45 and 58.)

TABLE 5
MILLIMOLAR ABSORPTIVITY OF HbO_2

λ (nm)	ϵ^{λ} (liters $mmol^{-1}$ cm^{-1})	Comments
930	0.31	Maximum
815	0.22	Isobestic with Hb
690	0.07	Minimum
592	2.13	Isobestic with HbCO
590.5	3.62	Isobestic with Hi
586	7.23	Isobestic with Hb
577	15.37	Maximum
572.5	13.50	Isobestic with HbCO
569	11.27	Isobestic with Hb
560	8.47	Minimum
549.5	12.06	Isobestic with HbCO
548.5	12.46	Isobestic with Hb
542	14.37	Maximum
540	14.27	Isobestic with HbCO
525.5	7.72	Isobestic with Hi
522	6.42	Isobestic with Hb
510	4.76	Minimum
506.5	4.81	Isobestic with Hb
497	5.16	Isobestic with HbCO
415	131.0	Maximum

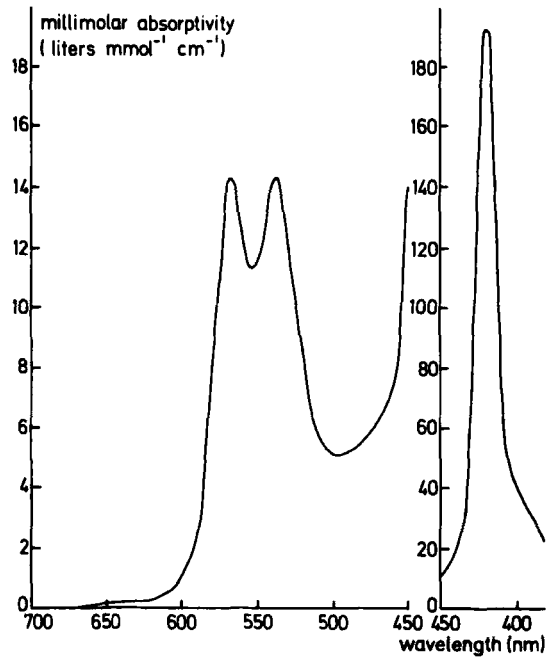

FIG. 13. Absorption spectrum of HbCO. (Based on data from refs. 45 and 58.)

TABLE 6
MILLIMOLAR ABSORPTIVITY OF HbCO

λ (nm)	ϵ^{λ} (liters mmol^{-1} cm^{-1})	Comments
585.5	3.79	Isobestic with Hi
579	8.86	Isobestic with Hb
572.5	13.50	Isobestic with HbO_2
568.5	14.31	Maximum
561.5	12.54	Isobestic with Hb
555	11.33	Minimum
549.5	12.06	Isobestic with HbO_2
547.5	12.37	Isobestic with Hb
540	14.27	Isobestic with HbO_2
539	14.36	Maximum
519.5	8.02	Isobestic with Hi
497	5.16	Isobestic with HbO_2
496	5.14	Minimum
420	192.0	Maximum

according to procedure 1. In Table 6, the millimolar absorptivity of HbCO is given for the maxima and minima in the spectral absorbance curve and for some isobestic points (cf. Fig. 10). Using procedure 2 and the HP8450A spectrophotometer, a millimolar absorptivity of 14.58 and 14.51 liters mmol^{-1} cm^{-1} was found in the maxima at λ = 568.6 and 539 nm and of 11.67 and 5.41 liters mmol^{-1} cm^{-1} in the minima at λ = 555 and 496 nm, respectively.

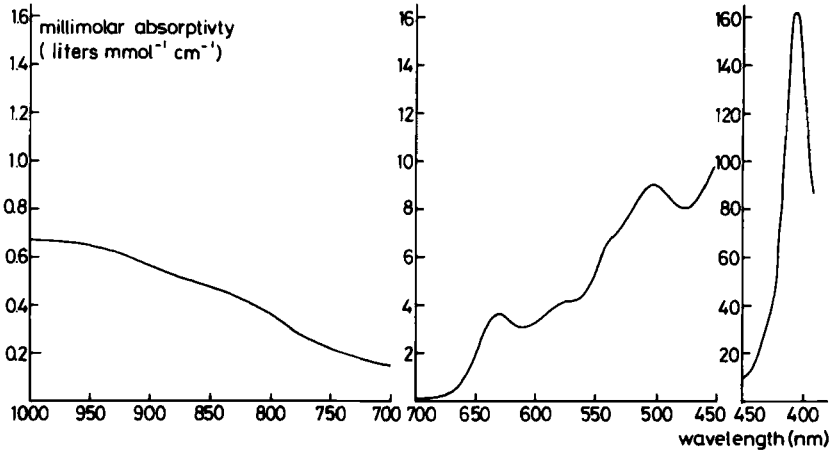

FIG. 14. Absorption spectrum of Hi. (Based on data from refs. 45 and 58.)

3.5. Hemiglobin (Methemoglobin; Hi)

The absorption spectrum of Hi is shown in Fig. 14 and, for the visible range, also in Fig. 10. The sample preparation for the data of Fig. 14 was according to procedure 1. In Table 7, the millimolar absorptivity of Hi is given for the maxima and minima in the spectral absorbance curve and for some isobestic points (cf. Fig. 10). The absorption spectrum of Hi is dependent on pH (cf. Fig. 9 in ref. 58), in contrast to those of the other hemoglobin derivatives, which are independent of pH over a wide range (pH 5.5–9.5), outside which denaturation occurs. The spectral absorptivity curves of Figs. 10 and 14 apply to pH 7.2. Using procedure 2 and the HP8450A spectrophotometer, a millimolar absorptivity of 3.90 and 9.25 liters $mmol^{-1}$ cm^{-1} was found in the maxima at $\lambda = 630$ and 500 nm and of 3.50 and 8.38 liters $mmol^{-1}$ cm^{-1} in the minima at $\lambda = 608$ and 476 nm, respectively.

3.6. Hemiglobincyanide (HiCN)

The spectral properties of this hemoglobin derivative have been extensively discussed in Section 2.1. The absorption spectrum is shown in Figs. 2, 3, and 10. The millimolar absorptivity in the maxima at $\lambda = 540$ and 421 nm is 10.99 (cf. Table 1) and 122.5 liters $mmol^{-1}$ cm^{-1} (45, 58), respectively. The millimolar absorptivity in the minimum at $\lambda = 504$ nm is 6.83 liters $mmol^{-1}$ cm^{-1}. It should be noted that $\epsilon^{540}/\epsilon^{504} = 10.99/6.83 = 1.609$ (cf. Section 2.5 and Table 3).

HiCN is the most stable of the hemoglobin derivatives that can be formed by the addition of an anion to Hi. The absorption spectra of two other

TABLE 7
Millimolar Absorptivity of Hi (pH 7.0–7.4)

λ (nm)	ϵ^{λ} (liters $mmol^{-1}$ cm^{-1})	Comments
690	0.13	Minimum
630	3.70	Maximum
608	3.06	Minimum
600	3.20	Isobestic with Hb
590.5	3.62	Isobestic with HbO_2
585.5	3.79	Isobestic with HbCO
528.5	7.71	Isobestic with Hb
525.5	7.72	Isobestic with HbO_2
519.5	8.02	Isobestic with HbCO
500	9.04	Maximum
476	8.04	Minimum
406	162.0	Maximum

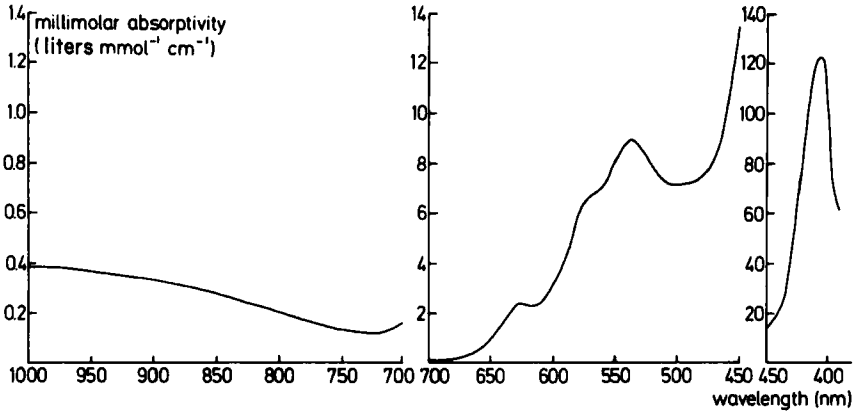

FIG. 15. Absorption spectrum of $HiNO_2$. (Based on data from ref. 45.)

hemoglobin derivatives of this kind ($HiNO_2$ and HiN_3) are dealt with in Section 3.7. A definite displacement series can be demonstrated for these compounds. The absorption spectra of HiF, $HiNO_2$, HiN_3, and HiCN change into each other upon the successive additon of F^-, NO_2^-, N_3^-, and CN^- ions to a solution of Hi.

3.7. HEMIGLOBINNITRITE ($HiNO_2$) AND HEMIGLOBINAZIDE (HiN_3)

The absorption spectra of $HiNO_2$ and HiN_3 are shown in Figs. 15 and 16. The sample preparation for the data in these figures was according to pro-

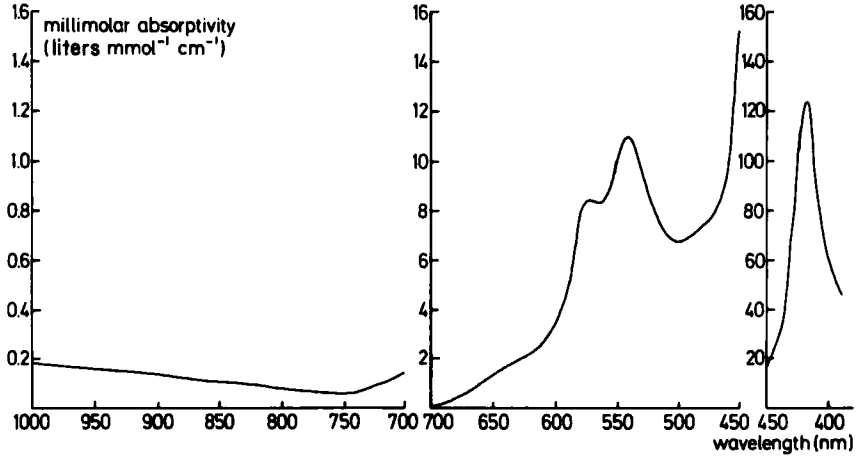

FIG. 16. Absorption spectrum of HiN_3. (Based on data from ref. 45.)

cedure 1 as described in Section 3.1. The shape of these two absorption spectra is not too different from that of HiCN. This also applies to the Soret band, where the millimolar absorptivity in the absorption maxima of $HiNO_2$, HiN_3, and HiCN is 123.5, 123.5, and 122.5 liters $mmol^{-1}$ cm^{-1}, respectively, although the wavelength of maximum absorption differs (λ = 406, 419, and 421 nm, respectively). The millimolar absorptivity of $HiNO_2$ at its two other absorption maxima, λ = 625 and 538 nm, is 2.48 and 9.02 liters $mmol^{-1}$ cm^{-1}, respectively. HiN_3 has maxima in the visible range at λ = 573 and 542 nm, with millimolar absorptivities of 8.51 and 10.95 liters $mmol^{-1}$ cm^{-1}, respectively.

It should be remembered that NO_2^- can enter the erythrocytes, whereas $Fe(CN)_6^{3-}$ cannot. Therefore, $NaNO_2$ is occasionally used to produce erythrocytes loaded with Hi. There has been some confusion in the literature as to the nature of the compound formed on the additon of $NaNO_2$ to whole blood or to a solution of hemoglobin (44). This is due to the fact that it has not always been recognized that the addition of an equimolar quantity of $NaNO_2$ leads to the formation of Hi, whereas the addition of excess $NaNO_2$ gives $HiNO_2$ (50).

Among the various reasons for which it was thought that HiN_3 could be easily substituted for HiCN in the standardized method for measuring c^*_{Hb} (cf. Section 2.2) was the near equality of the millimolar absorptivities of HiCN and HiN_3 at λ = 540 nm (ϵ^{540}_{HiCN} = 10.99; $\epsilon^{540}_{HiN_3}$ = 10.90). It has even been proposed that HiCN reference solutions be used for checking a routine HiN_3 method (60). The similarity between the two absorption spectra, however, is not strong enough to justify such a procedure (49).

3.8. Sulfhemoglobin (SHb), Sulfhemiglobin (SHi), and Sulfhemiglobincyanide (SHiCN)

In the sulfur-containing hemoglobin derivatives, the sulfur atom is bound to a pair of β-pyrrole carbon atoms at the periphery of a chlorin ring formed by saturation of the β–β double bond of the corresponding protoporphyrin IX of Hb. This structure explains the considerable departure of the absorption spectrum of SHb from that of Hb as well as the low affinity of SHb for O_2. For almost all hemoglobin derivatives which can be prepared starting from Hb, an SHb analog can be made. These compounds are of analytical rather than medical interest. The presence of SHbCO has been found once to be a source of error in the spectrophotometric determination of HbCO (56). SHiCN is formed when an SHb-containing blood sample is mixed with reagent solution for the determination of c^*_{Hb}, and is the cause of a very slight underestimation (cf. Section 2.2).

The absorption spectrum of SHb is shown in Fig. 17 and, for the visible

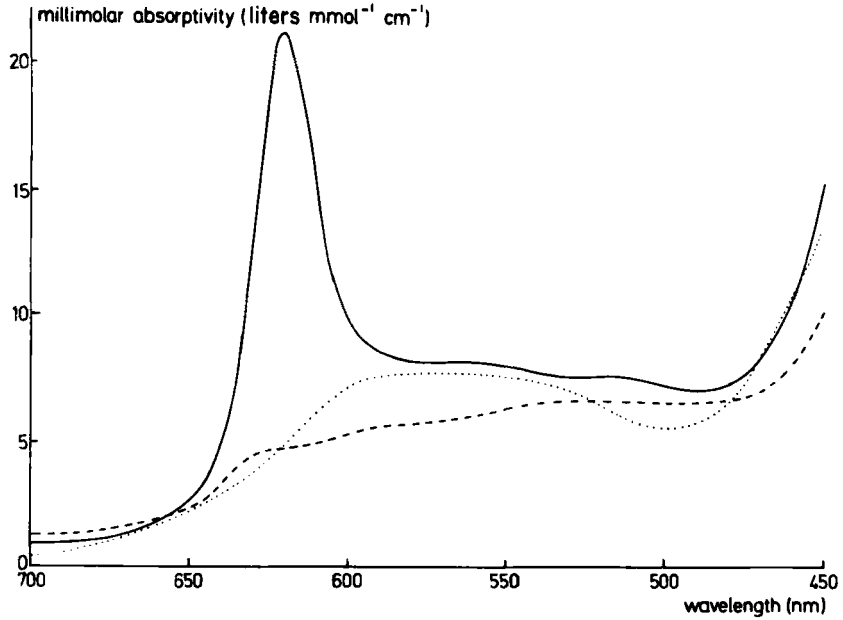

FIG. 17. Absorption spectra of SHb (—), SHi (---), and SHiCN (···). (Based on data from ref. 9.)

range, also in Fig. 10. Figure 17 shows, in addition, the absorption spectra of SHi and SHiCN. As SHb cannot be prepared in pure solution, a special procedure was necessary for the determination of its millimolar absorptivity. The SHb content of an erythrocyte suspension, prepared as described in Section 3.1, was determined by measuring the oxygen capacity before and after the formation of SHb. To this end, part of the oxygenated packed cells were mixed with the Na_2S/HCl solution of Section 3.1 (suspension A), and part with an oxygen-saturated 150 m*M* NaCl solution (suspension B). The erythrocyte concentrations of the two suspensions were made exactly equal. The oxygen content of the suspensions was then determined by means of the titrimetric method of Dijkhuizen *et al.* (10). For suspension B, c^*_{Hb} was measured using the standardized HiCN method, and O_2 capacity per g Hb (β_{O_2}) was calculated for both suspensions. The fraction of SHb in suspension A (F_{SHb}) then followed from

$$F_{SHb} = [\beta_{O_2}(B) - \beta_{O_2}(A)]/\beta_{O_2}(B) \qquad (6)$$

where $\beta_{O_2}(A)$ and $\beta_{O_2}(B)$ denote β_{O_2} for suspensions A and B, respectively.

After ultrasonic lysis of the erythrocyte suspensions, the ensuing solutions were diluted 50 times with 100 m*M* Tris buffer (pH 8.0) and filtered through a 0.45-μm Millipore filter. The absorbance of $\lambda = 620$ nm was measured

within 5 minutes with an Optica CF4 spectrophotometer in a cuvette with a lightpath length of 1.000 cm. The absorbance by SHb (A^{620}_{SHb}) was calculated with the equation

$$A^{620}_{SHb} = A^{620}_{A} - A^{620}_{B}(1 - F_{SHb}) \tag{7}$$

where A^{620}_{A} is the absorbance of the SHb/HbO_2 solution prepared from suspension A and A^{620}_{B} the absorbance of the HbO_2 solution prepared from suspension B. The millimolar absorptivity of SHb at $\lambda = 620$ nm (ϵ^{620}_{SHb}) then followed from

$$\epsilon^{620}_{SHb} = A^{620}_{SHb} f / F_{SHb} c^{*}_{Hb} l \tag{8}$$

where the dilution factor $f = 50$, the lightpath length $l = 1.000$ cm, and c^{*}_{Hb} is expressed in millimoles per liter.

For 11 samples with $F_{SHb} = 17.5$–24.3% and $c^{*}_{Hb} = 5.97$–8.92 mmol/liter, a mean value of $\epsilon^{620}_{SHb} = 20.82$ was found (SD = 1.49; SEM = 0.45) (9). These results are in fair agreement with those of Nichol and Morell (30) ($\epsilon^{620}_{SHb} = 21.5$) and only slightly higher than those of Siggaard-Andersen *et al.* (37) ($\epsilon^{620}_{SHb} = 18.1$, after recalculation to room temperature). All recent determinations give ϵ^{620}_{SHb} values which are much higher than the older ones such as those of Drabkin and Austin (12) ($\epsilon^{620}_{SHb} = 10.6$). The value of ϵ^{620}_{SHb} being determined, the ordinate of the spectral absorbance curves of SHb, SHi, and SHiCN recorded by means of the Aminco-Chance split-beam spectrophotometer (cf. Section 3.1) could be calibrated in absorptivity units (Fig. 17).

4. Determination of Hemoglobin Derivatives

Numerous spectrophotometric methods have been developed for the determination of hemoglobin derivatives in various mixtures and are described in the literature. For many problems, several solutions have been proposed. Therefore, the methods described in this section necessarily constitute a selection from many possibilities. The selection has been made on theoretical as well as practical grounds, not the least important of the latter being that the methods concerned yielded reliable results when tested in the authors' laboratories.

4.1. Theoretical Considerations

All methods for the spectrophotometric determination of hemoglobin derivatives depend on the validity of Lambert–Beer's law and all equations used to this end can be derived from the basic equations

$$A_X^\lambda = \epsilon_X^\lambda c_X l \tag{9}$$

and

$$A_X^\lambda = \log(I_0^\lambda / I_X^\lambda) \tag{10}$$

where A_X^λ is the absorbance of substance X at wavelength λ, I_0^λ is the amount of light of wavelength λ impinging on X, I_X^λ is the amount of light transmitted by X, ϵ_X^λ is the absorptivity of X at wavelength λ, c_X is the concentration of X, and l is the lightpath length. A is dimensionless; thus, when c is expressed in millimoles per liter and l in centimeters, ϵ is the millimolar absorptivity expressed in liters per millimole per centimeter.

When more than one light-absorbing component is present in a solution, the absorbance measured is the sum of the absorbances of all components present:

$$A^\lambda = A_1^\lambda + A_2^\lambda \cdots A_n^\lambda = \sum_{i=1}^{n} A_i \tag{11}$$

and

$$A^\lambda = \sum_{i=1}^{n} \epsilon_i^\lambda c_i l \tag{12}$$

When n components are present, the absorbance should be measured (at least) at n wavelengths to obtain (at least) n equations of the type of Eq. (12), from which the concentration of each component can be calculated. This procedure presupposes that the absorptivities of all components at all the wavelengths used (n^2 in number) and the lightpath length are exactly known. A straightforward application of this method has become feasible only recently (3, 74, 75). Formerly, an attempt was always made to reduce a multicomponent system to a series of two-component systems by various interconversions of the components.

The analysis of a two-component system through absorbance measurement at two wavelengths becomes much more simplified if, for one wavelength, an isobestic point is used. The two equations ensuing from Eq. (12) with $n = 2$ are

$$A^{\lambda_1} = \epsilon_1^{\lambda_1} c_1 l + \epsilon_2^{\lambda_1} c_2 l \tag{13}$$

and

$$A^{\lambda_2} = \epsilon_1^{\lambda_2} c_1 l + \epsilon_2^{\lambda_2} c_2 l \tag{14}$$

If λ_2 is the isobestic wavelength, $\epsilon_1^{\lambda_2} = \epsilon_2^{\lambda_2}$. Introducing the total concentration of the two light-absorbing components ($c = c_1 + c_2$) gives, through simple transformations (58),

$$c_2/c = [\epsilon_1^{\lambda 2} A^{\lambda 1}/(\epsilon_2^{\lambda 1} - \epsilon_1^{\lambda 1})A^{\lambda 2}] - [\epsilon_1^{\lambda 1}/(\epsilon_2^{\lambda 1} - \epsilon_1^{\lambda 1})] \tag{15}$$

or

$$F_2 = c_2/c = a(A^{\lambda 1}/A^{\lambda 2}) + b \tag{16}$$

With the aid of Eq. (16), the fraction of component 2 in the solution (F_2) can be calculated from the absorbance ratio $A^{\lambda 1}/A^{\lambda 2}$ if the constants a and b are known. These constants can easily be determined by a series of measurements of the absorbance ratio of solutions exclusively containing component 1 or component 2 (58). It should be noted that neither the exact value of the absorptivities nor the exact layer thickness needs to be known. In spite of the advent of practical multicomponent methods, two-wavelength methods utilizing an isobestic point are still suitable for many purposes, e.g., for the determination of the oxygen saturation and the carbon monoxide saturation of human blood (vide infra).

Instead of selecting an isobestic point for one of the wavelengths in a method for analyzing a two-component system, one can select a pair of wavelengths at which the absorptivities of one of the components are equal ($\epsilon_1^{\lambda 1} = \epsilon_1^{\lambda 2}$). Subtracting Eq. (14) from Eq. (13) then gives

$$A^{\lambda 1} - A^{\lambda 2} = (\epsilon_2^{\lambda 1} - \epsilon_2^{\lambda 2})c_2 l \tag{17}$$

or

$$c_2 = (A^{\lambda 1} - A^{\lambda 2})/(\epsilon_2^{\lambda 1} - \epsilon_2^{\lambda 2})l = k(A^{\lambda 1} - A^{\lambda 2}) \tag{18}$$

Using Eq. (18), the concentration of component 2 in the solution (c_2) can be calculated from the absorbance difference $A^{\lambda 1} - A^{\lambda 2}$ if the constant k is known. This constant can be determined by means of a series of measurements of the absorbance difference of solutions with a known concentration of component 2. These can, of course, be pure solutions of component 2, of which the concentration can be determined after conversion to HiCN. It should be noted that k is dependent on l, so that the same cuvette or exactly equal cuvettes should be used in all measurements.

The analysis a two-component system by absorbance measurement at a single wavelength requires either an exact knowledge of the absorptivities of the two components, the lightpath length, and the sum of the concentration of the two components, or a second measurement, after conversion of the component to be measured to another hemoglobin derivative. The equation to be used with the first of these methods follows from Eq. (13) when it is taken into account that $c = c_1 + c_2$. Thus,

$$F_2 = c_2/c = A^{\lambda}/cl\ [1/(\epsilon_2^{\lambda} - \epsilon_1^{\lambda})] - [\epsilon_1^{\lambda}/(\epsilon_2^{\lambda} - \epsilon_1^{\lambda})] \tag{19}$$

where c follows from c_{Hb}^*, taking into account the dilution factor, if any, used in preparing the solution of which the absorbance is actually measured.

When a conversion method is used, two absorbance measurements are made at the same wavelength. This yields two equations, analogous to Eq. (13):

$$A_1^\lambda = \epsilon_1^\lambda c_1 l + \epsilon_2^\lambda c_2 l \tag{20}$$

$$A_2^\lambda = \epsilon_1^\lambda c_1 l + \epsilon_3^\lambda c_2 l \tag{21}$$

where ϵ_2^λ is the absorptivity of component 2 before, and ϵ_3^λ the absorptivity after, conversion to another hemoglobin derivative. Subtraction of Eq. (21) from Eq. (20) yields

$$A_1^\lambda - A_2^\lambda = (\epsilon_2^\lambda - \epsilon_3^\lambda) c_2 l \tag{22}$$

and

$$c_2 = (A_1^\lambda - A_2^\lambda)/(\epsilon_2^\lambda - \epsilon_3^\lambda) l \tag{23}$$

Dividing Eq. (23) by the sum of the concentration of the two components in the mixture gives

$$F_2 = c_2/c = (A_1^\lambda - A_2^\lambda)/(\epsilon_2^\lambda - \epsilon_3^\lambda) lc = (A_1^\lambda - A_2^\lambda)/(A_3^\lambda - A_4^\lambda) \tag{24}$$

where $(A_3^\lambda - A_4^\lambda)$ is the absorbance difference occurring when all the hemoglobin in the solution is subject to conversion. (It should be noted that the lower indices to A refer to the sequence of the absorbance measurements, whereas those to ϵ and c refer to the components.) Therefore, if the absorbance difference after first changing all the hemoglobin into component 2 can also be measured, the fraction of component 2 can be calculated from the absorbance measurement without knowing the absorptivities and the light-path length and without the necessity of determining any constants.

4.2. Determination of the Oxygen Saturation (S_{O_2})

The oxygen saturation of the blood (S_{O_2}) is defined as the amount of oxygen actually bound by the hemoglobin in the erythrocytes in a certain volume of blood divided by the oxygen capacity, i.e., the maximum amount of oxygen which can be bound by the hemoglobin in the erythrocytes in the same volume of blood. Thus

$$S_{O_2} = c_{HbO_2}/(c_{HbO_2} + c_{Hb}) \tag{25}$$

The oxygen saturation should not be confused with the oxyhemoglobin fraction (F_{HbO_2}):

$$F_{HbO_2} = c_{HbO_2}/c_{Hb}^* \tag{26}$$

Only in the theoretical case when no dyshemoglobins (hemoglobin derivatives which have permanently or temporarily lost the ability to combine

reversibly with oxygen under physiological conditions) are present does S_{O_2} equal F_{HbO_2}. Usually, S_{O_2} is slightly higher than F_{HbO_2}.

For the determination of S_{O_2}, two-wavelength methods with an isobestic point are excellently suited. In our previous review (58), we described two methods in which the absorbance measurements were made at $\lambda = 560$ and 522 nm and $\lambda = 560$ and 506 nm, respectively (cf. Tables 4 and 5). The use of wavelengths at which the absorptivity is rather high, instead of the classic "oximeter band" around $\lambda = 650$ nm, was advocated on the basis of the consideration that possible turbidity due to plasma protein has less influence in regions of high hemoglobin absorptivity, and because of the wish to avoid the use of the isobestic point in the near infrared, which then appeared to be quite elusive. This isobestic point is the obvious choice for combination with a measurement in the region around $\lambda = 650$ nm because the difference in absorptivity at the two wavelengths should not be too big (Fig. 18). Only then is it possible to keep both the absorbance measurements in the range of near maximum photometric accuracy by proper selection of the lightpath length (cf. Fig. 2 in ref. 58).

A practical disadvantage of measuring at $\lambda = 560/522$ or 560/506 nm is the necessity of using a very small lightpath length ($l = 0.01$ cm). Yet, there are more substantial reasons for preferring a method with measurement in the red/near-infrared region for the determination of S_{O_2}. First, as Mook *et al.*

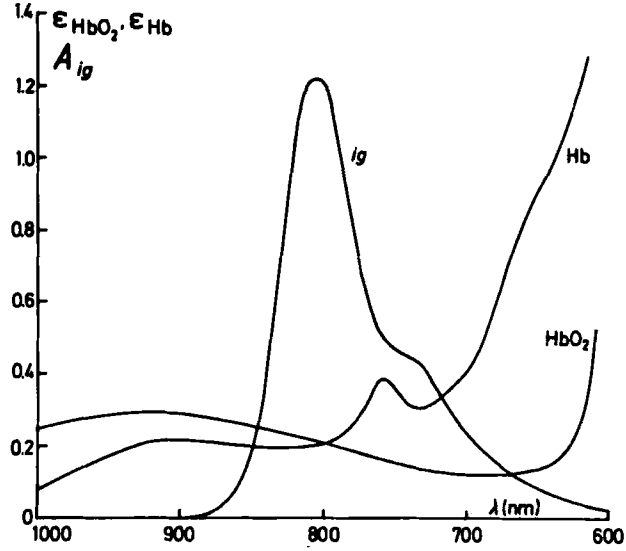

FIG. 18. Absorption spectra of HbO_2 and Hb in the red and near-infrared spectral region. Also shown is the spectral absorbance curve of a 5 mg/liter indocyanine green solution, measured in a cuvette with a lightpath length of 1.000 cm. [From Mook *et al.* (25).]

(28) have demonstrated, the sensitivity of the measurement, expressed as dA/dS_{O_2}, the change in absorbance per unit change in oxygen saturation, is much higher between $\lambda = 600$ and 730 nm than in any other region of the visible spectrum. This easily compensates for the somewhat stronger influence of possible turbidity due to plasma protein in this region than at shorter wavelengths. Second, it has been shown that the difficulty of determining the isobestic point in the near infrared is solely the result of the use of $Na_2S_2O_4$. When the blood is deoxygenated by tonometry with N_2/CO_2, the isobestic point is consistently found at $\lambda = 800$ nm, as shown in Fig. 18. For 14 blood samples of healthy human donors, Mook *et al.* (25) obtained $\lambda = 799.9 \pm 0.96$ nm (SD). Third, in the red/near-infrared region, the pair of wavelengths can be chosen in such a manner that the influence of the possible presence of HbCO and Hi in the blood sample on the measured value of S_{O_2} is small.

An excellent choice for the determination of S_{O_2} is the wavelength pair $\lambda = 680$ and 800 nm. The absorbance measurements are best carried out with a lightpath length of 0.2 cm. The following procedure can be recommended. An arterial or venous blood sample is collected in a glass syringe containing an amount of heparin sufficient to prevent coagulation. For an S_{O_2} measurement, 2 ml of blood is transferred anaerobically to a 2-ml syringe, which contains a glass pellet or a small metal ring for mixing and of which the dead space has been filled with a 10% solution of a nonionic detergent (cf. Section 2.2). After thoroughly mixing the sample and discarding the first three drops, the hemolysate is injected with the help of a blunt needle into a plan-parallel glass cuvette with a lightpath length of 0.200 cm. The cuvette is completely filled, closed with a glass cover, and placed in the spectrophotometer. The absorbance is measured at $\lambda = 680$ and 800 nm, using as a blank a similar cuvette filled with water. S_{O_2} is calculated with an equation of the type of Eq. (16). The values of the constants a and b in this equation are determined by measuring A^{680}/A^{800} for some completely oxygenated and deoxygenated blood samples. This yields absorbance ratios corresponding to $S_{O_2} = 1$ and 0, respectively. By substituting these in Eq. (16), two equations are obtained from which a and b can be solved.

This calibration was carried out by means of an Optica CF4 grating spectrophotometer, using completely oxygenated and deoxygenated blood of six humans and eight dogs. Substitution of the values for a and b in Eq. (16) results in

$$S_{O_2} = -0.3819(A^{680}/A^{800}) + 1.633 \tag{27}$$

These values of a and b are slightly different from those which would have resulted from the substitution $\epsilon^{680}_{HbO_2}$, ϵ^{680}_{Hb}, and $\epsilon^{800}_{HbO_2/Hb}$ in Eq. (15). The cause of this difference is that the absorptivities were determined for par-

tially purified hemoglobin solutions, whereas in the direct determination of a and b, the absorbance of hemolysates is measured without any clearing procedure. The latter is obviously the correct way to determine these constants because, in a practical spectrophotometric method for the determination of S_{O_2}, no preparatory steps other than hemolysis should be necessary. Moreover, calculating a and b from the absorptivities gives quite inaccurate results in this spectral region where the absorptivities are very low and, consequently, the uncertainty in the available values is considerable.

Some data on the influence of HbCO and Hi present in a blood sample on the S_{O_2} values obtained by measuring A^{680}/A^{800} are shown in Table 8. It appears that the error, caused by such amounts of HbCO and Hi and which may go undetected for some time, is not large, except for Hi in the lower oxygen saturation range. In the latter case, an appreciable overestimation of S_{O_2} occurs. This will, however, seldom cause problems in the clinical application of the method.

The absorption maximum of indocyanine green, the dye commonly used in measuring cardiac output by means of the dye dilution method, coincides with the isobestic point of Hb and HbO_2 at $\lambda = 800$ nm, as shown in Fig. 18. This coincidence was actually the main reason for the introduction of the dye, because it allows the recording of dye dilution curves without interference due to variation in oxygen saturation. The high absorptivity of indocyanine green at $\lambda = 800$ nm (19), however, precludes the use of this wavelength for the determination of S_{O_2} when the blood contains the dye. It has been shown that by using the wavelength pair $\lambda = 660$ and 860 nm, S_{O_2} can be determined virtually independent of the presence of indocyanine green (25). The procedure is the same as with the 680/800 method. However, Eq. (16) does not hold well for the relationship between the absorbance ratio A^{680}/A^{860} and S_{O_2} since no isobestic point is used. A slightly more complicated equation can be easily derived from Eq. (12), taking $n = 2$ (25,

TABLE 8
ERRORS IN THE DETERMINATION OF OXYGEN SATURATION[a]

	Measured values of S_{O_2} (%)			
S_{O_2} (%)	$F_{HbCO} = 5\%$	$F_{HbCO} = 10\%$	$F_{Hi} = 5\%$	$F_{Hi} = 10\%$
100	99.7	99.3	99.7	99.5
80	79.7	79.3	81.5	82.8
60	59.7	59.3	63.2	66.2
40	39.7	39.3	44.9	49.5

[a] Absorbance measurement at $\lambda = 680$ and 800 nm; errors caused by presence of HbCO and Hi.

58). The 660/860 method may be useful for the determination of S_{O_2} in blood samples taken during cardiac catheterization, when dye dilution curves are being made.

4.3. Determination of the Carbon Monoxide Fraction (F_{HbCO})

In the description of the relationship between carbon monoxide and hemoglobin, the same distinction between saturation and fraction can be made as in the case of oxygen. Carbon monoxide saturation can be defined as the amount of carbon monoxide actually bound by the hemoglobin in the erythrocytes in a certain volume of blood divided by the carbon monoxide capacity, i.e., the maximum amount of carbon monoxide which can be bound by the hemoglobin in the erythrocytes in the same volume of blood. Thus

$$S_{CO} = c_{HbCO}/(c_{HbCO} + c_{HbO_2} + c_{Hb}) \tag{28}$$

This equation differs from Eq. (25) in that HbO_2 is treated as a hemoglobin derivative that can bind carbon monoxide, whereas in the definition of oxygen saturation, HbCO is treated as a hemoglobin derivative that cannot bind oxygen. From a chemical point of view this distinction may seem arbitrary but, physiologically, it does make sense. The affinity of hemoglobin for CO is so much greater than for O_2 that the P_{O_2} difference between the arterial and the venous blood has virtually no influence on the amount of HbCO in the blood. Consequently, the amount of hemoglobin that is in the form of HbCO is at least temporarily lost for the oxygen transport function. HbCO is thus correctly termed a dyshemoglobin (cf. Section 4.2) and should preferably be expressed as a fraction [cf. Eq. (29)]. That HbCO even impedes oxygen transport through its influence on the O_2 affinity of the rest of the hemoglobin in the blood is not relevant in this context.

The first choice to be made in designing a two-wavelength method for the determination of the HbCO fraction in blood is the two-component system to be used: HbCO/Hb or HbCO/ HbO_2. In our previous review (58), we described for each system a two-wavelength method with an isobestic point. The method utilizing the system HbCO/Hb has the advantage that the absorbances can be measured in a diluted solution, so that a 1.00-cm cuvette can be used. The method is reliable and accurate enough for most clinical purposes. It is carried out as described in ref. 58.

In the following, a method is described utilizing the system HbCO/HbO_2. This method has also already been dealt with in ref. 58, but it has been critically evaluated since. As the method presupposes the system HbCO/HbO_2, the fresh, heparinized blood sample is first oxygenated by rotating it for 5 minutes in a small, open, cylindrical tonometer, flushed with

oxygen just before use. It has been shown that this procedure is sufficient to completely saturate the Hb present with O_2, while the influence on the HbCO in the sample is insignificant for HbCO fractions < 40% (8).

One milliliter of the tonometered blood is transferred to a 1-ml syringe, which contains a glass pellet or a small metal ring for mixing and of which the dead space has been filled with a 10% solution of a nonionic detergent (cf. Section 2.2). If less blood is available, the volume ratio of blood/detergent solution can be changed, but a correspondingly lower detergent concentration should be used. It has been shown that a twofold dilution is inconsequential for the HbCO fraction (58). After thoroughly mixing the sample and discarding the first three drops, the hemolysate is injected with the help of a blunt needle into a plan-parallel glass cuvette with a lightpath length of 0.100 cm. A 0.095-cm glass plate is inserted into the cuvette, leaving a lightpath length of 0.005 cm (cf. Fig. 13 in ref. 58 and Fig. 2 in ref. 75). The cuvette is placed in the spectrophotometer and the absorbance is measured at λ = 562 and 540 nm, using as a blank a similar cuvette filled with water. The isobestic point is at λ = 540 nm (Fig. 10; Tables 5 and 6).

For calculating the HbCO fraction from the absorbance ratio A^{562}/A^{540}, the following equation is used, which is of the type of Eq. (16):

$$F_{HbCO} = 3.215(A^{562}/A^{540}) - 1.923 \tag{29}$$

The constants $a = 3.215$ and $b = -1.923$ are based on the data of Fig. 19. These data were collected by measuring A^{562}/A^{540} of 46 human blood samples containing various amounts of HbCO. The blood was obtained from 22 healthy donors and contained heparin as anticoagulant. Part of the blood was oxygenated, part of it was equilibrated with a CO-containing gas mixture, and samples with various HbCO fractions were prepared by mixing various volumes of HbO_2- and HbCO-containing blood. The HbCO fractions were determined by a titrimetric method (8), which is a modification of the method for the determination of the oxygen content of blood described in detail in ref. 10.

The value of A^{562}/A^{540} for CO-free blood, following from the regression line of Fig. 19, is 0.598, which agrees well with the results of the direct measurement, ranging from 0.593 to 0.603. It is also in excellent agreement with the results of another series of measurements of A^{562}/A^{540} of blood after tonometry with pure O_2 for 150 minutes (7): 0.599 ± 0.004 (n = 38). The zero point of the determination of F_{HbCO} has thus been firmly established. It should be noted that the possible presence of Hi in the blood sample has practically no influence on the absorbance ratio, $(\epsilon^{562}/\epsilon^{540})_{Hi}$ being ~ 0.610. The presence of some 20% Hi would simulate less than 1% HbCO. The spread in the value of A^{562}/A^{540} (±0.004, corresponding with ±1.6% HbCO) will therefore be due to variations in the light-absorbing and -scattering properties of plasma constituents and erythrocyte stromata.

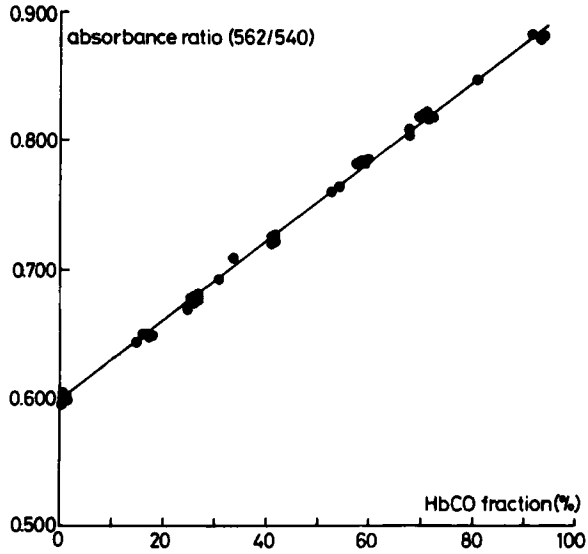

FIG. 19. Absorbance ratio A^{562}/A^{540} plotted against the corresponding HbCO fraction determined with the titrimetric method. The equation of the regression line is $A^{562}/A^{540} = 0.3118F_{HbCO} + 0.59756$. The correlation coefficient $r = 0.9991$. [From Dijkhuizen *et al.* (8).]

The value of A^{562}/A^{540} for blood with 100% HbCO, following from the regression line of Fig. 19, is 0.909. Direct determination, however, yielded a slightly lower value: 0.900 ± 0.003 ($n = 84$). No cause for this discrepancy has yet been found. If the constants of Eq. (29) are calculated from the absorbance ratios directly measured for blood, with $F_{HbCO} = 0$ and 1 (0.599 and 0.900, respectively), $a = 3.322$ and $b = -1.990$. The error in the determination of F_{HbCO} caused by the use of these constants is insignificant: instead of $F_{HbCO} = 0$, 10, 20, 30, and 40%, we get −0.3, 10.0, 20.4, 30.7, and 41.0%, respectively.

4.4. Determination of the Hemiglobin Fraction (F_{Hi})

In our previous review (58), we described a two-wavelength method with an isobestic point for the determination of Hi in the system Hi/HbO_2 and, also, the CN^- addition method according to Evelyn and Malloy (13). The latter method has the advantage that it is an absolute method in the sense that it is not dependent on constants which have been previously determined by measuring samples of known concentration. The method is invalidated by the presence of SHb (9), which is not too serious a disadvantage because of the infrequent occurrence of this hemoglobin derivative and its easy spectrophotometric detectability (cf. Fig. 10). In our experience, the

CN^- addition method gives reliable results for $F_{Hi} < 30\%$; with higher Hi fractions there is a progressive overestimation. Also, because of its simplicity, the method is well suited for routine application in the clinical chemical laboratory.

In the CN^- addition method, four diluting solutions are used. All solutions contain 0.05% of a nonionic detergent (cf. Section 2.2.), 27.50 m*M* Na_2HPO_4, and 13.16 m*M* KH_2PO_4. Solutions 2 and 3 contain, in addition, 3.84 m*M* KCN and 3.04 m*M* $K_3Fe(CN)_6$, respectively. Solution 4 contains, in addition, 3.84 m*M* KCN as well as 3.04 m*M* $K_3Fe(CN)_6$. One-half milliliter blood is added to 25 ml of each of the four solutions. In the first solution, the Hi present in the sample will remain unchanged. In the second solution, all Hi present becomes converted to HiCN. In the third solution, all hemoglobin present in the sample (except SHb) is converted to Hi, and in the fourth solution, all hemoglobin present (except SHb) is converted to HiCN. The absorbance of the four solutions is measured at $\lambda = 630$ nm, with either $l = 1.00$ cm or $l = 4.00$ cm, according to the absorbance level, using as a blank a similar cuvette filled with water. The Hi fraction is then calculated with an equation of the type of Eq. (24), in which $F_2 = F_{Hi}$, $\lambda = 630$ nm, and A_1, A_2, A_3, and A_4 are the absorbances measured for the blood diluted with solutions 1, 2, 3, and 4, respectively.

4.5. Multicomponent Analysis of Hemoglobin Derivatives

The straightforward application of a set of equations of the type of Eq. (12) for the simultaneous determination of n hemoglobin derivatives in a sample is based on the assumptions (1) that it is known which hemoglobin derivatives may be present, (2) that Lambert–Beer's law is valid for all components in the mixture, (3) that Lambert–Beer's law also applies to the actual mixture as it is presented for the absorbance measurements, (4) that the available absorptivity values are applicable to the actual measuring conditions, and (5) that the lightpath length is exactly known. Strictly speaking, the fourth assumption is simply another formulation of the third. One of the problems in the application of this method is that the measurements should preferably be carried out with simple hemolysates. The mere necessity of keeping the oxygen saturation of the sample constant precludes most clearing procedures. Therefore, the samples of which the absorbance is measured are necessarily turbid. Thus, it is a matter of semantics whether it is said that Lambert–Beer's law does not strictly apply to the solution because of the presence of light-scattering material, or that the available absorptivity values are not strictly valid for the hemoglobin derivatives as present in the hemolysate.

It has been shown that by simple anaerobic filtration through cotton wool, the hemolysate can be made clear enough for multicomponent analysis using absorptivity values as presented in Section 3. This technique has been applied in the simultaneous determination of Hb, HbO_2, HbCO, Hi, and SHb with a conventional spectrophotometer (75). In this method, measurements are made at five carefully selected wavelengths and the concentrations of the hemoglobin derivatives are calculated by solving a set of five equations of the type of Eq. (12). Such a system is called an exactly determined system, since there are just as many equations as there are unknowns. When the absorbance is measured over a considerable spectral range, either continuously or in very small steps, many more equations are obtained than there are unknowns. Such a system is called an overdetermined system. The HP8450A UV/Vis spectrophotometer (Hewlett-Packard, Palo Alto, California) is equipped with a computer program for multicomponent analysis in an overdetermined system. This procedure is now used for the simultaneous determination of the five hemoglobin derivatives previously mentioned (76).

To this end, a new set of spectral absorptivity curves is being made in our laboratories by measuring hemolyzed blood containing known concentrations of single hemoglobin derivatives (in the case of SHb, known mixtures of SHb and HbO_2). The ensuing collection of absorptivity curves are stored on magnetic tape and can be loaded into the microcomputer whenever the spectrophotometer is to be used for the analysis of a blood sample. Thus, in this procedure, the absorptivities are determined under exactly the same conditions as those under which the measurements of the unknown mixtures are made. The spectral absorptivity curves of Fig. 10 are the result of such a procedure. In the near future, it will also be possible to utilize these spectra for a similar procedure using conventional spectrophotometers, since these are being increasingly equipped with a microcomputer.

In the following, a five-wavelength method is described for the simultaneous determination of Hb, HbO_2, HbCO, Hi, and SHb, which can be carried out by means of a conventional spectrophotometer (75). The fresh, heparinized blood sample is transferred without any air contact from the syringe in which it was collected to a 2-ml glass syringe containing a mixing ball (ϕ = 5 mm), with the dead space filled with a 5% solution of a nonionic detergent (cf. Section 2.2). After blood and detergent solution have been thoroughly mixed, a filter unit containing a piece of cotton wool is fixed onto the syringe. After flushing filter space and needle by discarding about 10 drops, two cuvettes (l = 0.200 and 0.100 cm) are filled with hemolysate. A 0.093-cm plan-parallel glass plate (Hellma Benelux, The Hague, The Netherlands) is inserted into the 0.100-cm cuvette. The absorbance is measured at λ = 760 and 620 nm with l = 0.200 cm and at λ = 577, 569, and 500 nm with l = 0.007 cm. In all measurements, a similar cuvette filled with water is used as a blank in the reference channel.

A spectral band width of 1 nm or less should be used. The wavelength and absorbance scales of the spectrophotometer should be checked as described in Section 2.4. The lightpath length of the cuvettes should be known exactly and can be checked as described in Section 3.1. The concentrations of the hemoglobin derivatives present in the sample are calculated from the absorbances by matrix calculation using the absorptivities of Table 9. In this calculation, the dilution of the blood with detergent solution and possible slight differences between the cuvettes in the measuring channel and the reference channels should be taken into account. An example of a program for making the calculation with a desk-top calculator (HP9845A) is given in ref. 75.

By means of an Optica CF4 spectrophotometer, the five-wavelength method has been compared for all five components with specific methods for the determination of each of these components (75). For the oxygen saturation, a comparison was made with the two-wavelength method described in Section 4.2 (λ = 680 and 800 nm), using 22 blood samples from three healthy humans. Different values of S_{O_2} were obtained by tonometry with various O_2/N_2 mixtures. The results are shown in Fig. 20. The deviation of the five-wavelength method with respect to the 680/800 method was 1.2 ± 2.3% S_{O_2} (SD).

For the carboxyhemoglobin fraction, a comparison was made with the two-wavelength method described in Section 4.3 (λ = 562 and 540 nm), using 32 blood samples from 7 healthy humans. Different values of F_{HbCO} were obtained by mixing in various proportions HbO_2-containing blood made by tonometry with O_2 and HbCO-containing blood made by tonometry with a CO-containing gas mixture. The results are shown in Fig. 21. The deviation of the five-wavelength method with respect to the 562/540 method was 1.2 ± 1.7% F_{HbCO} (SD).

For the hemiglobin fraction, a comparison was made with the method described in Section 4.4 (CN^- addition method), using 16 blood samples

TABLE 9
MATRIX OF MILLIMOLAR ABSORPTIVITIES

	ϵ^λ (liters $mmol^{-1}$ cm^{-1})				
λ (nm)	Hb	HbO_2	HbCO	Hi	SHb
500	4.09	5.05	5.35	9.04	7.20
569	11.27	11.27	14.27	4.10	8.10
577	9.40	15.37	10.00	4.10	8.10
620	1.23	0.24	0.33	3.35	20.80
760	0.43	0.13	0.03	0.24	1.04

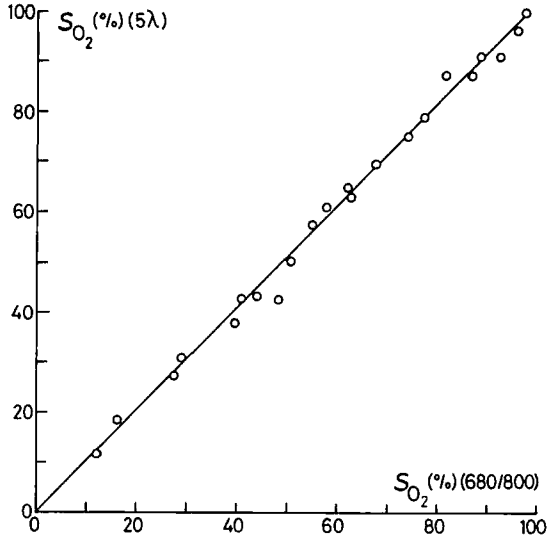

FIG. 20. Oxygen saturation (S_{O_2}) by 5 λ method plotted against S_{O_2} by 680/800 method. Equation of the regression line is S_{O_2} (5 λ) = 1.03 × S_{O_2} (680/800) − 0.32. Correlation coefficient r = 0.993. [From Zwart *et al.* (75).]

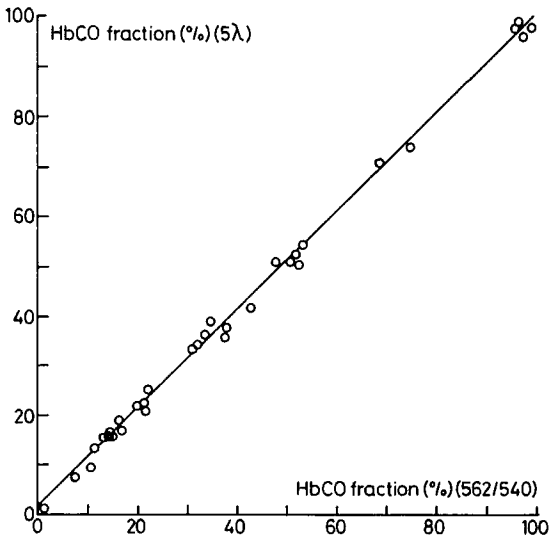

FIG. 21. Carboxyhemoglobin fraction (F_{HbCO}) by 5 λ method plotted against F_{HbCO} by 562/540 method. Equation of the regression line is F_{HbCO} (5 λ) = 0.99 × F_{HbCO} (562/540) + 1.45. Correlation coefficient r = 0.996. [From Zwart *et al.* (75).]

from 4 healthy humans. In order to prepare samples with various amounts of Hi, the blood was hemolyzed by the addition of a drop of undiluted Sterox SE and distributed to several test tubes to which various volumes of a 90 m*M* solution of hexacyanoferrate(III) were added. The tubes were kept for 90 minutes at room temperature before the absorbance of the samples was measured. The results are shown in Fig. 22. The deviation of the five-wavelength method with respect to the CN^- addition method was $-0.4 \pm 0.7\%$ F_{Hi} (SD). This comparison had to be limited to the range $F_{Hi} = 0$–30% because of the progressive overestimation of F_{Hi} by the CN^- addition method when $F_{Hi} > 30\%$ (cf. Section 4.4). Some additional dilution experiments indicated that the five-wavelength method gives correct values for F_{Hi} at least up to 80%.

For the sulfhemoglobin fraction, a comparison was made with a single-wavelength method utilizing an equation of the type of Eq. (19) and meeting the requirements to be fulfilled for the proper use of this equation (cf. Section 4.1). SHb-containing blood was prepared as described in Section 3.1. One-half milliliter blood was then added to 25 ml of a solution of 0.05% Sterox SE in phosphate buffer of pH 7.4 and the absorbance of this solution measured at $\lambda = 620$ nm, with $l = 1.00$ or 4.00 cm, according to the absorbance level. The results are shown in Fig. 23. The difference of the

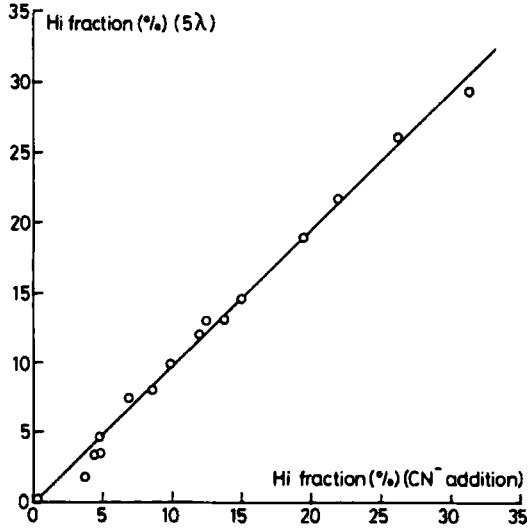

FIG. 22. Hemiglobin fraction (F_{Hi}) by 5 λ method plotted against F_{Hi} by CN^- addition method. Equation of the regression line is F_{Hi} (5 λ) = 0.99 × F_{Hi} (CN^-) − 0.31. Correlation coefficient $r = 0.994$. [From Zwart *et al.* (75).]

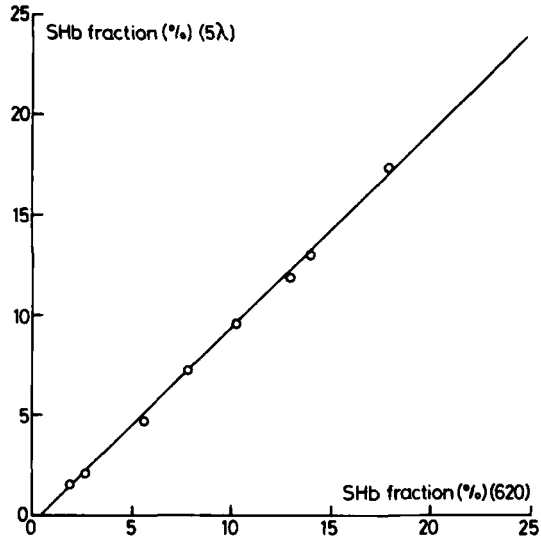

FIG. 23. Sulfhemoglobin fraction (F_{SHb}) by 5 λ method plotted against F_{SHb} by 620 method. Equation of the regression line is F_{SHb} (5 λ) = 0.99 × F_{SHb} (620) − 0.52. Correlation coefficient r = 0.998. [From Zwart *et al.* (75).]

five-wavelength method with respect to the 620 method was −0.6 ± 0.2% F_{SHb} (SD). The comparative measurements had to be limited to the range F_{SHb} = 0–25% because test samples with more than 25% SHb cannot be obtained by the technique used.

The total hemoglobin concentration of 22 samples from 5 healthy humans was determined with the HiCN method and the result compared with c^*_{Hb} calculated by adding the concentrations of all hemoglobin derivatives present in the sample. The results are shown in Fig. 24. The deviation of the five-wavelength method with respect to the HiCN method was −0.06 ± 0.15 g/dl (SD). The equality of c^*_{Hb} (5 λ) and c^*_{Hb} (HiCN) affords an easy opportunity for interparametric quality control (54). It is sound practice to supplement each determination of hemoglobin derivatives by means of the five-wavelength method with a determination of c^*_{Hb} as HiCN.

A four-wavelength method for the determination of Hb, HbO_2, HbCO, and Hi has been realized using an automated spectrophotometer, the IL282 CO-Oximeter (Instrumentation Laboratory Inc., Lexington, Maine) (3). The four wavelengths (λ = 535.0, 585.2, 594.5, and 626.6 nm) are obtained by means of four interference filters, each selecting a particular line from the emission spectrum of a Tl–Ne hollow cathode lamp. The advantage of this construction is that the wavelength setting is extremely stable and no regular

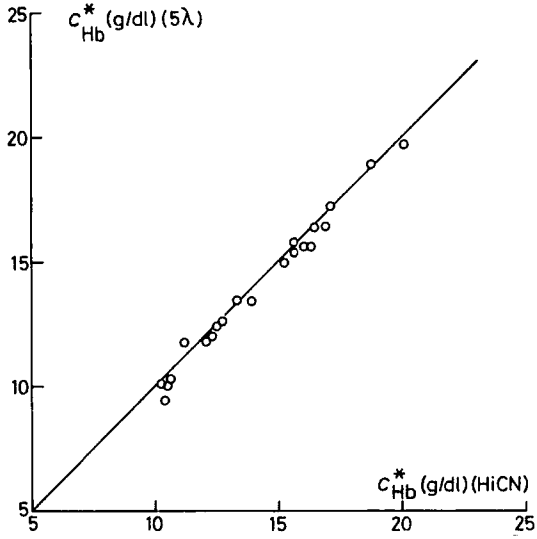

FIG. 24. Total hemoglobin concentration (c^*_{Hb}) by 5 λ method plotted against c^*_{Hb} by HiCN method. Equation of the regression line is c^*_{Hb} (5 λ) = 1.00 × c^*_{Hb} (HiCN) − 0.07. Correlation coefficient r = 0.988. [From Zwart *et al.* (75).]

wavelength calibration is necessary. A disadvantage is that the wavelengths to be used cannot be chosen freely at the most suitable points in the absorption spectra of the components. The IL282 CO-Oximeter was compared with the five-wavelength method described above and proved to be accurate for the determination of the HbO_2 and HbCO fractions and for c^*_{Hb}. The determination of Hi was somewhat less accurate. Even SHb could be detected, but not quantitated exactly (74).

5. Concluding Remarks

The internationally standardized HiCN method for the determination of the total hemoglobin concentration in human blood has now reached a stage of general acceptance, at least, as a reference method. The absorption spectrum of HiCN and the exact value of the millimolar absorptivity at λ = 540 nm have been established beyond any doubt, and the validity of Lambert–Beer's law for HiCN solutions has been confirmed. The production, control, and distribution of the international HiCN reference solution have become a matter of routine; no undesired incidents have occurred since the production started in 1964. A hypoosmolar $K_3Fe(CN)_6$/KCN solution, buff-

ered with KH_2PO_4 to pH 7.2 and containing a nonionic detergent, has been proven to be the most suitable reagent solution and is widely accepted. The remaining uncertain points—the influence of the possible presence of SHb and the freeze-destruction of the reagent solution—have at last been elucidated: the influence of SHb is insignificant and the freeze-destruction of the reagent can be prevented by simple means.

The exact knowledge of the millimolar absorptivity of HiCN at $\lambda = 540$ nm constitutes a reliable reference point for the absorptivities of other hemoglobin derivatives. Because all hemoglobin derivatives except those containing sulfur can be converted into HiCN, any absorbance measured for any hemoglobin derivative can be easily related to this reference point. Thus, there remain few problems in the determination of spectral absorptivity curves of most hemoglobin derivatives.

Accurate knowledge of the spectral absorptivity curves of the common hemoglobin derivatives is the first requirement for the determination of these compounds in a mixture by multicomponent analysis. The calculating facilities necessary for the practical application of multicomponent analysis are now rapidly becoming available to the clinical chemical laboratory. Thus, it seems obvious that better and easier methods for multicomponent analysis of an increasing number of hemoglobin derivatives will be developed in the near future. Most wanted are improved tricks for suppressing turbidity in the hemolysates without changing the composition of the mixture of hemoglobin derivatives. For measuring oxygen saturation, the established two-wavelength methods utilizing an isobestic point, carried out either by means of a general purpose spectrophotometer or with a special instrument, will certainly be used for many years to come. Besides, reflection oximetry will remain an attractive alternative, especially for measuring *in vivo* (18, 26, 27, 33, 67).

In some cases, it is useful to calculate the oxygen content of the blood ($c^*_{O_2}$) after the total hemoglobin concentration (c^*_{Hb}), the oxygen saturation (S_{O_2}), and the dyshemoglobin fractions (F_{dysHb}) (cf. Section 4.2) have been determined. The following equation applies:

$$c^*_{O_2} = S_{O_2}\beta_{O_2}c^*_{Hb}(1 - F_{dysHb}) + \alpha_{O_2}P_{O_2} \tag{30}$$

where β_{O_2} is the oxygen capacity per gram hemoglobin, α_{O_2} the solubility of O_2 in whole blood, and P_{O_2} the oxygen tension. For $c^*_{Hb} = 150$ g/liter, $\alpha_{O_2} = 0.2201$ ml/liter·kPa at 37°C (6), and at an arterial P_{O_2} of 14 kPa, only 3.08 ml O_2 is in physical solution in 1 liter of blood. This is only 1.5% of the total amount of O_2 in the arterial blood and can be neglected for all practical purposes. Thus

$$c^*_{O_2} = S_{O_2}\beta_{O_2}c^*_{Hb}(1 - F_{dysHb}) = \beta_{O_2}c_{HbO_2} \tag{31}$$

Multicomponent analysis as described in Section 4.5 can give the oxyhemoglobin concentration (c_{HbO_2}) directly. As to the value of β_{O_2}, it seems obvious to use the theoretical value of $22.4 \times 10^{-3}/16114.5 = 1.390$ ml/g because, in Eq. (31), appropriate corrections for the dyshemoglobins have been made. However, in most cases this will probably give a slight overestimation. There seems to be a small, as yet unidentified, inactive hemoglobin fraction. In a series of 36 human blood samples, Dijkhuizen *et al.* (7) found this fraction to be $0.9 \pm 1.2\%$ (SD), but in two cases it was as high as 3.9 and 4.3%, respectively. As long as this hemoglobin fraction has not been identified and included in the multicomponent analysis, the error caused by it has to be taken for granted.

Several blood gas analyzers are equipped with a program for calculating S_{O_2} from P_{O_2}, pH, and P_{CO_2}. The calculation is based on the standard oxygen dissociation curve, and the temperature coefficient and the Bohr factors, stored in the data processor. However, there are other factors influencing the oxygen affinity of hemoglobin which cannot easily be taken into account. One of these is the 2,3-diphosphoglycerate (2,3-DPG) content of the erythrocytes. Another source of error is the presence of types of hemoglobin with an oxygen affinity different from that of HbA, of which HbF is, of

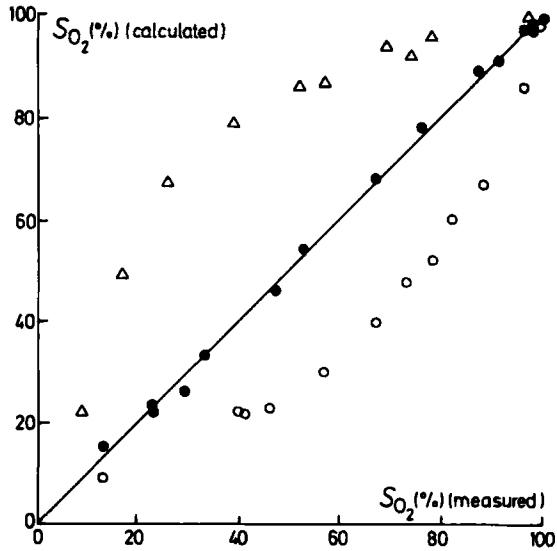

FIG. 25. Influence of 2,3-DPG content of erythrocytes on oxygen saturation (S_{O_2}) values calculated from P_{O_2} and pH (Corning 175). S_{O_2} measured with a special purpose photometer (Radiometer OSM2). Dots: fresh donor blood with normal 2,3-DPG content. Open circles: outdated bank blood, containing very little 2,3-DPG. Triangles: blood with about four times the normal 2,3-DPG content. [From Oeseburg *et al.* (32).]

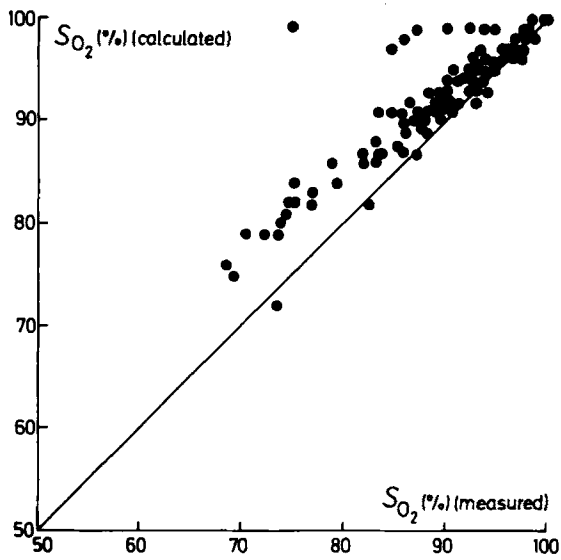

FIG. 26. Calculated (Corning 175) versus measured S_{O_2} (Radiometer OSM2) for 128 blood samples from critically ill patients. [From Oeseburg *et al.* (32).]

course, the most frequently occurring. Figure 25 shows the considerable influence of 2,3-DPG. For fresh donor blood with a normal 2,3-DPG content, there is an excellent agreement between the calculated and measured values of S_{O_2}. When the 2,3-DPG content is very low, the calculated values are much lower than the measured values. When the 2,3-DPG concentration is very high (about 4 times the normal [2,3-DPG]/[Hb_4] ratio), the calculated values are much too high. A comparison of calculated and measured values of S_{O_2} for 128 blood samples of critically ill patients (Reanimation Centre, University Hospital, Groningen, The Netherlands) showed considerable differences, the calculated values usually being too high (Fig. 26). This clearly demonstrates that the oxygen saturation should not be calculated, but should be determined by one of the several excellent methods available for this purpose.

6. Appendix

Definition of terms used in quality control.

Accuracy: agreement between the best estimate of a quantity and its true value.

Precision: agreement between results of replicate measurements in iden-

tical material; the quantitative measure is the standard deviation or the coefficient of variation.

Bias: numerical difference (+ or −) between the best estimate and the true value; equivalent term: systematic error.

As true values are essentially unknown, values from reference methods are used instead. It may happen that a method yields results with a very good reproducibility (high precision), but with a considerable systematic error (low accuracy). It should therefore be kept in mind that equality does not guarantee quality.

For obtaining more insight into the random error (standard deviation) σ_r and the systematic error (bias) σ_s, the Youden plot may be helpful. For this purpose, two samples with concentrations c_1 and c_2 are analyzed in n laboratories. The values obtained for c_1 are plotted against the values obtained for c_2 as shown in Fig. 27.

When only random errors are present, the chance of getting a deviation $+\Delta c$ is equal to the chance of getting a deviation $-\Delta c$. The points (c_1,c_2) from the various laboratories will thus be evenly spread around the point $(\bar{c}_1,\bar{c}_2)$. This result is a circular distribution. When a systematic error is present, the circular distribution changes to an elliptic one. The greater the systematic error, the narrower the ellipse.

This can be quantitated in the following way (Fig. 27). When $\sigma_s/\sigma_r = 0$, 50% of all points are found in quadrants B and D (circular distribution). When $\sigma_s/\sigma_r = 1$, 2, and 3, about 70, 80, and 90%, respectively, of all points

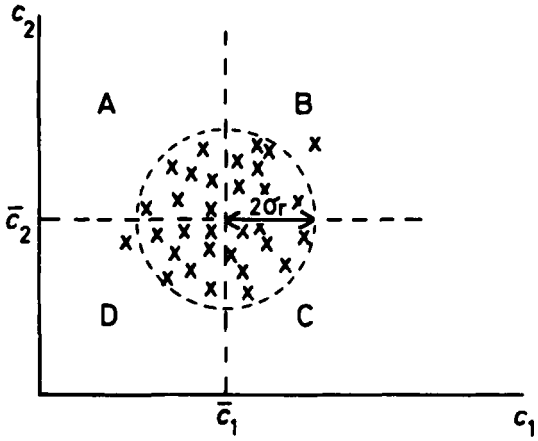

FIG. 27. Youden plot of concentration measurements c_1 and c_2 of two samples of the same compound by various laboratories. The circular distribution shown signifies the absence of systematic errors. The greater the number of laboratories making systematic errors, the more points are found in quadrants B and/or D.

are in quadrants B and D. When $\sigma_s/\sigma_r \geqslant 4$, all points are in quadrants B and D and the ellipse approximates a straight line.

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