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Haemoglobin Interference in the Bichromatic Spectrophotometry of NAD(P)H at 340/380 nm

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Summary: The negative bias observed in the NADPH-based bichromatic measurement of glucose in haemolysates (da Fonseca-Wollheim, F., Heinze, K.-G. & Liss, E. (1992) Temperature-dependent matrix effect in the direct enzymatic measurement of blood glucose, this journal *30*, 371–375) is caused by shifts in the UV absorbance of haemoglobin which affect the absorbance difference $\Delta A_{340/380\,nm}$. In model experiments with haemoglobin solutions, spectral changes resulting in decreases of the absorbance at 340 nm and/or increases at 380 nm were found to occur for the following three reasons:

1. Oxidation of haemoglobin-O_2 with formation of Fe(III) derivatives. Methaemoglobin formation is accelerated by lowering the pH, raising the temperature from 25 to 37 °C or by adding organic phosphates (inositol hexakisphosphate, ATP). At pH 6, addition of plasma increases the rate of methaemoglobin formation, while at pH values > 7, haemoglobin-O_2 is stabilised. The oxidation of haemoglobin-O_2 in the presence of sodium lauryl sulphate is also accompanied by a decrease of $\Delta A_{340/380\,nm}$. The haemichromes formed in this reaction exhibit stable UV light absorptivity.
2. Increase in the temperature of the haemoglobin-O_2 solution. It is shown that the temperature-induced shifts in the haemoglobin-O_2 absorptivity are reversible and that similar changes occur with the chemically more stable cyanomethaemoglobin.
3. Deoxygenation of haemoglobin-O_2 at low pO_2. Theoretically, the variation of factors influencing the pO_2 (0.5) such as temperature, pH and allosteric effectors can also lead to changes in $\Delta A_{340/380\,nm}$.

The shifts in the UV absorbance of haemoglobin solutions are largely independent of the presence of NAD(P)H. In methods based on bichromatic spectrophotometry of NAD(P)H with reference readings in the 374–380 nm region, the possibility of spectral interference by haemoglobin should be considered. Blank readings should be performed only after reaching temperature equilibrium. If haemolysates are to be analysed, the selection of a reference wavelength beyond the *Soret* region may be necessary.

Introduction

Interference by haemoglobin in NAD(P)H-dependent spectrophotometric methods for the measurement of substrate or enzyme activity concentrations has been documented only in a very few cases (1, 2). Apart from a possible interference by other erythrocyte constituents, it is generally expected that the analysis of moderately haemolytic serum samples or even of haemolysates is possible if appropriate sample blank readings are performed. However, at very high haemoglobin concentrations in the order of 143 μmol/l[1]) a suppression of NADH absorbance and a proportional decrease of the measured transketolase[2]) activ-

[1]) Substance concentration of the haemoglobin monomer, M_r = 16110.

[2]) Enzymes:
Sedoheptulose-7-phosphate : *D*-glyceraldehyde-3-phosphate glycolaldehydetransferase, EC 2.2.1.1
Hexokinase, ATP : *D*-hexose 6-phosphotransferase, EC 2.7.1.1.

ity by 10% were found (3). Recently, in the measurement of glucose in haemolysates with an NADPH-dependent hexokinase[2] procedure, a constant bias of −0.43 mmol/l was observed in the presence of approximately 20 µmol/l haemoglobin, using bichromatic spectrophotometry at 37 °C (4). We have suggested that temperature-induced changes in the UV absorbance spectrum of haemoglobin at 340 and/or 380 nm may cause this previously unknown and reagent-independent matrix effect (5). Preliminary experiments performed in the present study have shown that

(a) the oxidation of haem groups,

(b) temperature-induced changes in the absorptivity of haemoglobin-O_2,

(c) deoxygenation of haemoglobin-O_2

can lead to absorbance shifts in the UV region. The observed inconstancy of the background absorbance of samples containing haemoglobin should be considered as a source of analytical variation in NAD(P)H-dependent procedures, especially if bichromatic spectrophotometry at 340/380 nm or similar wavelengths is applied.

Methods

Instrumentation

For the registration of absorbance spectra or monochromatic readings of absorbances at fixed wavelengths a UVIKON 810 spectrophotometer connected to a recorder 21 and an LS Printer 48 (Kontron Instruments, Zurich, Switzerland) was used. The cuvette holder was thermostatically controlled and the temperature of solutions within the cuvettes was checked by a digital thermometer with a resolution of 0.1 °C. Spectral scans of haemoglobin derivatives between 250 and 700 nm (fig. 2 and 5) were performed with a Beckman DU 650 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, U.S.A.). Bichromatic measurements at 340/380 nm were performed with an Abbott VP Series II Bichromatic Analyzer (Abbott Laboratories, Irving, TX, U.S.A.). Blood haemoglobin concentrations were measured with a Coulter Model STKS haematologic apparatus (Coulter Electronics Inc., Hialeah, FL, U.S.A.). Oximetry was performed with a Corning 2500 CO-Oximeter (Corning Medical and Scientific, Corning Glass Works, Medfield, MA, U.S.A.).

Reagents

NADH disodium salt, cat. No. 107727, NADPH tetrasodium salt, cat. No. 107824, ATP disodium salt, cat. No. 519979 and 2,3-diphosphoglyceric acid pentacyclohexylammonium salt, cat. No. 15178 were obtained from Boehringer Mannheim GmbH, Mannheim, Germany. Inositol hexakisphosphoric acid dipotassium salt, cat. No. P-5681, Digitonin, cat. No. D-1407 and Triton X 100, cat. No. X-100 were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Lauryl sulphate sodium salt, cat. No. 20760 was from Serva, Heidelberg, Germany. The other chemicals used were reagent grade.

Haemoglobin preparations

Venous blood anticoagulated with 50 × 10^3 IU/l sodium heparin (Liquemin, Hoffmann-La Roche AG, Grenzach-Wyhlen, Germany) was repeatedly obtained during the study from an apparently healthy male non-smoker in the fasting state. Oximetry was performed after 5-min mixing of the blood sample with an excess of atmospheric air at room temperature. Typical results were: haemoglobin 9.62 mmol/l, fractions of oxyhaemoglobin, carbon monoxide haemoglobin and methaemoglobin 0.981, 0.018 and 0.0, respectively. Blood cells were separated by centrifugation at 1700 *g* and washed three times with a solution of 0.15 mol/l NaCl at 4 °C, discarding the buffy coat. The cells were suspended in a solution of 0.15 mol/l NaCl, resulting in a haemoglobin concentration of approximately 10 mmol/l. Aliquots of the plasma obtained during the procedure and 100 µl portions of the washed cells were stored for some days at −38 °C. For use in the experiments, the frozen cell sediment was thawed by adding a sufficient volume of 0.05 mol/l sodium phosphate buffer, pH 7.5, at room temperature to prepare a solution with 0.52 mmol/l oxyhaemoglobin. Haemolysis was complete. The oxyhaemoglobin solution was cleared by centrifugation at 12000 *g* for 2 min and was kept at 0 °C until use. Very dilute haemoglobin concentrations were determined by a manual cyanomethaemoglobin procedure (6). Stroma-free haemoglobin was prepared according to l.c. (7). PD-10 minicolumns (Pharmacia, Uppsala, Sweden) were used to separate haemoglobin from low-molecular-mass constituents. Apart from oxyhaemoglobin, the final preparation contained only small fractions of methaemoglobin (0.003) or carbon monoxide haemoglobin (0.018). For the preparation of methaemoglobin, an aliquot of the haemoglobin preparation was oxidized with a small excess of ferricyanide followed by gel chromatography (8). The methaemoglobin obtained by this procedure contained < 0.02 parts of reduced haem (9) as confirmed by spectrophotometric measurement at 630 nm (10).

Procedures

Absorbance spectra

The absorbance spectra of haemoglobin derivatives were read using solutions in 0.05 mol/l sodium phosphate buffer. A quartz cuvette of 10 mm path length was used. The spectral band width was always 2 nm. The values of buffer pH, haemoglobin concentration or temperature within the cuvettes are given in the figures.

Monitoring of absorbance changes at 340/380 nm and constant temperature

Five hundred µl of 0.05 mol/l sodium phosphate buffer of various pH values, with the additions shown in the figures, were placed in a segment of the multicuvette of the Abbott VP Bichromatic Analyzer for a 10-min period of preincubation at the chosen temperature. Four s before the first reading of $\Delta A_{340/380\,nm}$ 20 µl of a solution containing 520 µmol/l haemoglobin were added by multipette (Eppendorf Gerätebau Netheler-Hinz, Hamburg, Germany). Fifty µl of silicone oil AK 350 (Wacker Chemie, Munich, Germany) were placed on the upper surface during the first revolution to avoid concentration effects due to evaporation. Further spectrophotometric readings were taken at 2-min intervals for 30 min.

Monitoring of absorbance changes at 340/380 nm with changing temperature

With an empty multicuvette segment in the measuring position, the Abbott VP Bichromatic Analyzer was started in the "read mode" to print out the absorbance at 340/380 nm at 4-s intervals. Three hundred µl of a solution containing 50 µmol/l of

haemoglobin in 0.05 mol/l sodium phosphate buffer, pH 7.5, were transferred from a test tube incubated at 25 °C to the multicuvette kept at 37 °C in the water bath of the analyser. After approximately 2 min the mixture within the cuvette reached the preset temperature. The course of the absorbance changes was followed for 480 s starting when the mixture was added. Analogous experiments were performed for the temperature change 37 to 25 °C (see fig. 8).

Deoxygenation of haemoglobin solutions

Three ml of 50 µmol/l oxyhaemoglobin in 0.05 mol/l sodium phosphate buffer pH 7.5 was rotated under humidified N_2 for 10 min in an IL 237 Tonometer (Instrumentation Laboratory Lexington, MA, U.S.A.) at room temperature. An aliquot of the treated solution was transferred anaerobically to a cuvette sealed by a rubber diaphragm for the registration of the absorbance spectrum under N_2 at 25 °C. Oxygen saturation of haemoglobin before and after tonometry was measured according to l.c. (11).

Results

1. Spectral changes in the UV absorbance of haemoglobin solutions due to haem oxidation

1.1 *Spectral shift caused by methaemoglobin formation*

If solutions of haemoglobin-O_2 are incubated at 37 °C, methaemoglobin is formed by autoxidation (12). The continuous autoxidation is demonstrated by the time-dependent increase in the absorbance at 630 nm. At the same time the absorbance increases above 348 nm, and decreases below this wavelength (fig. 1). The difference spectra (methaemoglobin against haemoglobin-O_2) show that the absorbance changes are pH-dependent and most pronounced in the *Soret* region (fig. 2). Accordingly, any conversion of haemoglobin-O_2 into methaemoglobin will induce interference in the spectrophotometric measurement of substrates or enzyme activities based on the formation or consumption of NAD(P)H. At a given rate of methaemoglobin formation in the test solution one has to expect a relatively slight negative interference in monochromatic spectrophotometry at 340 nm. If the formation/disappearance of NAD(P)H is monitored by bichromatic spectrophotometry (13) with a reference reading at 380 nm a marked decrease of the signal

$$A_{340\,nm} - A_{380\,nm} = \Delta A_{340/380\,nm}$$

will occur due to methaemoglobin formation, because the positive deviations at 380 nm and the negative deviations at 340 nm cause effects in the same direction.

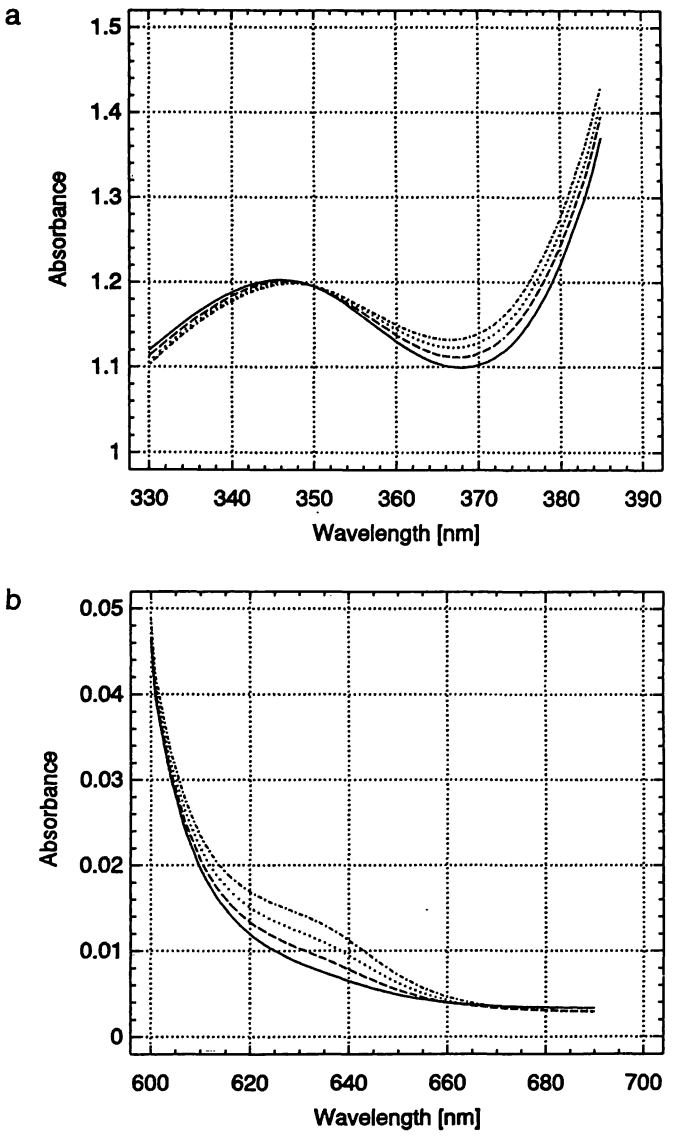

Fig. 1a, b. Course of spectral changes with 50 µmol/l oxyhaemoglobin in phosphate buffer, pH 6, at 37 °C. Spectra at 0 (——), 20 (----); 40 (···) and 60 (·—·—·) min after reaching temperature equilibrium.

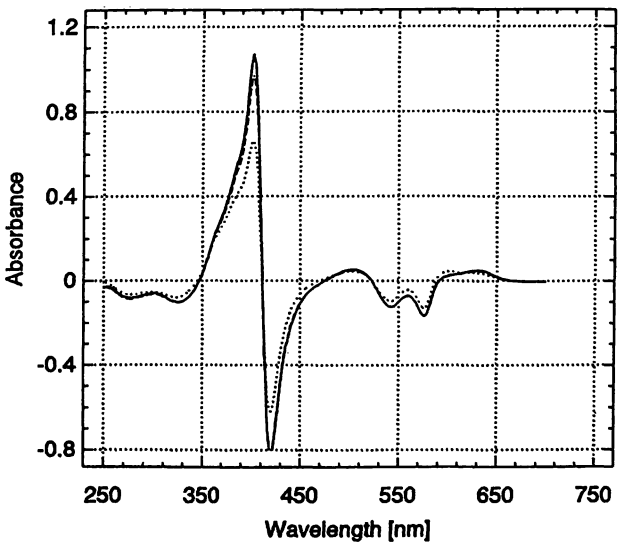

Fig. 2. Difference spectra (methaemoglobin against oxyhaemoglobin) obtained with 15 µmol/l haemoglobin at 25 °C. pH 6 (——), 7 (----), or 8 (···).

1.2 *Influences on methaemoglobin formation*

The rate of decrease in $\Delta A_{340/380\,nm}$ was investigated in model experiments with haemoglobin solutions obtained by freezing and thawing of washed erythrocytes (see "Methods"). $\Delta A_{340/380\,nm}$ decreased constantly over 30 min (fig. 3). The decrease per 30 min at 37 °C was maximal at pH 6 and minimal at pH 8 (fig. 4). Addition of plasma (volume fraction 0.04) accelerated methaemoglobin formation at pH 6 but inhibited it at pH values ≥ 7. ATP (1 mmol/l) caused a moderate, 0.5 mmol/l inositol hexakisphosphate a strong acceleration of the process (fig. 4), while 1 mmol/l 2,3 bisphosphoglycerate was ineffective (data not shown). Raising the temperature from 25 to 37 °C led on average to a 2.7-fold increase of the methaemoglobin formation rate. With 0.05 mmol/l NADH or NADPH, the rate of decrease in $\Delta A_{340/380\,nm}$ was always higher than in absence of cosubstrate (fig. 4), although a correction was made for the spontaneous degradation of NADH and NADPH (14) observed with buffer blanks as described in figure 8.

1.3 *Change of $\Delta A_{340/380\,nm}$ due to formation of haemichromes*

In the presence of sodium lauryl sulphate, haemoglobin is converted to haemichromes (15). The absorbance spectra of haemichrome formed in 2.08 mmol/l sodium lauryl sulphate and of haemoglobin-O_2 differ

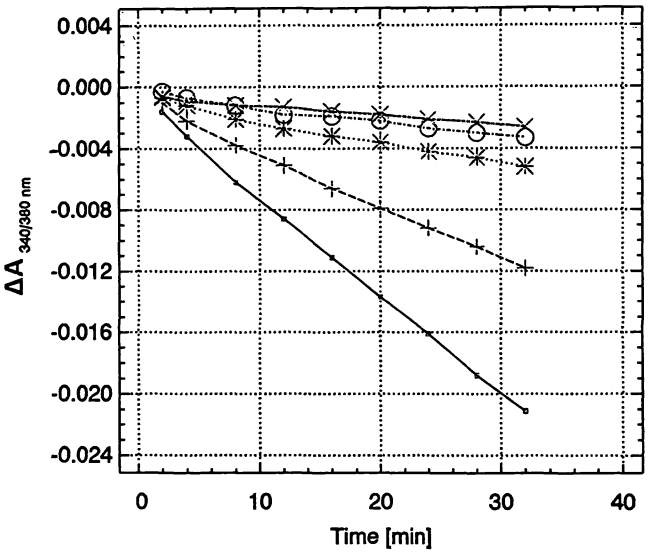

Fig. 3. Changes in $\Delta A_{340/380\,nm}$ with time during incubation of 20 µmol/l oxyhaemoglobin at 37 °C pH 6.0 (——), 6.5 (— — —), 7.0 (···), 7.5 (· — · — ·), 8.0 (----).

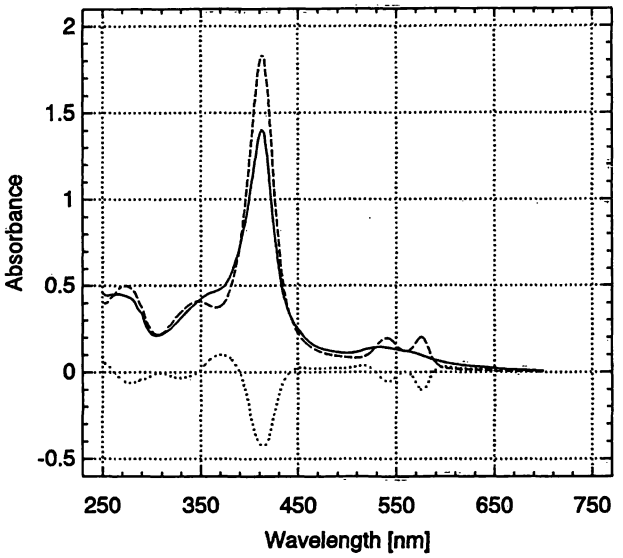

Fig. 5. Absorbance spectra of haemichromes formed in 2.08 mmol/l sodium lauryl sulphate (——) and of oxyhaemoglobin (----). Additionally the difference spectrum (haemichrome against oxyhaemoglobin) is shown (···). Haemoglobin concentration 15 µmol/l, pH 7.5, T = 25 °C.

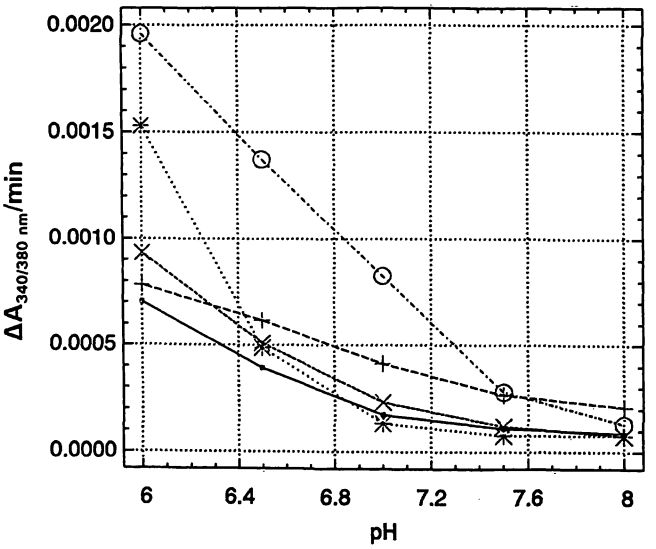

Fig. 4. Decreases of $\Delta A_{340/380\,nm}$ during incubation of 20 µmol/l oxyhaemoglobin at pH 7.5 and 37 °C.
No additions (▫—▫), 0.05 mmol/l NADH (+ — +), plasma (1 : 26) (* — *), 0.5 mmol/l inositol hexakisphosphate (○—○), 1 mmol/l ATP (x—x).

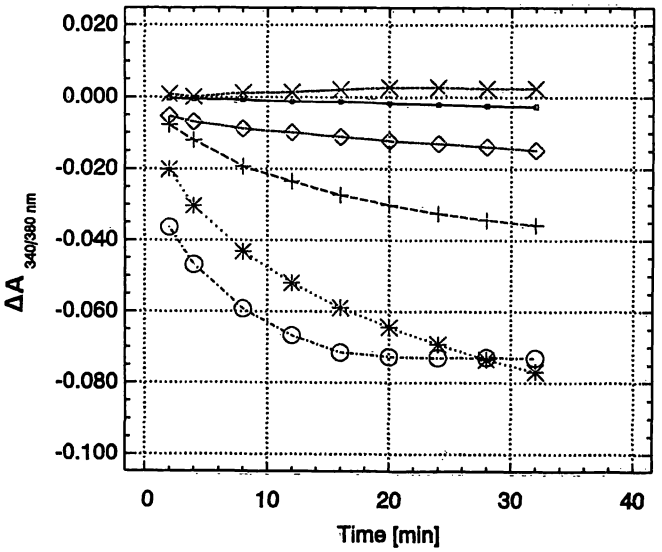

Fig. 6. Changes in $\Delta A_{340/380\,nm}$ with respect to the initial reading during incubation of 20 µmol/l oxyhaemoglobin in the presence of detergent, pH 7.5, 37 °C.
▫—▫ no additions, sodium lauryl sulphate at 0.13 (+ — +), 0.26 (* — *), 0.52 (○—○) or 1.04 (x—x) mmol/l/l; 0.6 g/l Triton X-100 (◇ — ◇).

markedly in the UV range (fig. 5). The course of the changes in $\Delta A_{340/380\,nm}$ observed at various concentrations of sodium lauryl sulphate is shown in figure 6. In the presence of 1.11 mmol/l sodium lauryl sulphate, a nearly stable UV absorbance was achieved immediately, while, at lower concentrations, the conversion into haemichromes progressed in a concentration-dependent manner. If plasma was added, higher concentrations of sodium lauryl sulphate were necessary to obtain stable absorbance. In the presence of 0.6 g/l Triton X-100, the drift of $\Delta A_{340/380\,nm}$ was accelerated (fig. 6). With digitonin at concentrations between 2–18 mg/l, the constancy of $\Delta A_{340/380\,nm}$ was as good as in the absence of a haemolysing agent (data not shown).

2. Temperature-induced changes in the UV absorbance of haemoglobin solutions

Changes in the absorptivity of haemoglobin derivatives as a function of temperature have been described for the visible part of the spectrum (16) and for the region around 290 nm (17). Corresponding data for the 340–380 nm region do not appear to be available. When the temperature of a solution of haemoglobin-O_2 in 0.05 mol/l sodium phosphate buffer, pH 7.5, was increased from 25 to 37 °C within two minutes, the UV absorbance spectrum shifted positively above 354 nm with maximal deviations at 372 nm (fig. 7). Below 354 nm there were slight negative changes. At 37 °C, the absorbance spectrum was unstable, due to methaemoglobin formation (fig. 7). With continuous bichromatic monitoring (fig. 8), a rapid decrease of $\Delta A_{340/380\,nm}$ was observed during the first two minutes until temperature equilibrium was reached (see "Methods"). Afterwards, the indicated $\Delta A_{340/380\,nm}$ decreased at a constant rate, due to methaemoglobin formation (see above). Conversely, positive changes of $\Delta A_{340/380\,nm}$ were observed when the temperature was reduced from 37 to 25 °C. The temperature-induced shifts were nearly the same in the presence of reduced cosubstrate after correction by appropriate buffer blanks. Similar temperature-induced changes in $\Delta A_{340/380\,nm}$ occurred when a solution of cyanomethaemoglobin was used (fig. 8).

3. Changes in the UV absorbance spectrum of haemoglobin solutions following deoxygenation

Due to the differences between the molar absorptivities of oxy- and deoxyhaemoglobin in the UV region (18), a marked shift of the absorbance occurred when the oxygen saturation of haemoglobin was changed from 0.93 to 0.35 by short tonometry with N_2 (fig. 9).

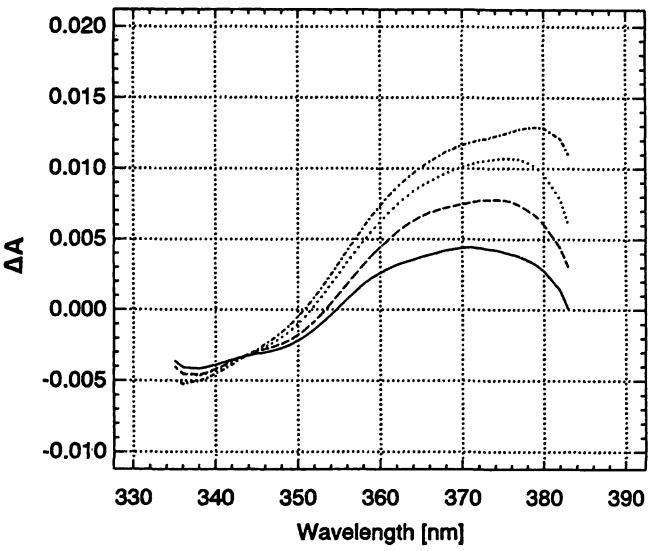

Fig. 7. Shifts of the UV absorbance with 50 µmol/l oxyhaemoglobin at 37 °C as compared to 25 °C; pH 7.5. Shifts after 2 min (——), 10 min (----), 20 min (···), or 30 min (·—·—·).

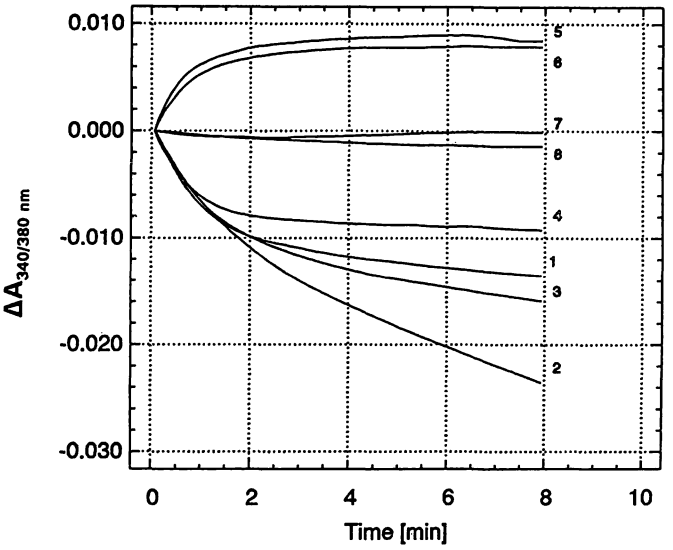

Fig. 8. Temperature-induced changes in $\Delta A_{340/380\,nm}$ with 50 µmol/l haemoglobin derivative and/or 50 µmol/l reduced cosubstrate. Negative changes occurred on heating from 25 to 37 °C (1–4, 7 and 8), positive on cooling from 37 to 25 °C (5–6).
Oxyhaemoglobin at pH 7.5 (1) or 6.5 (2);
oxyhaemoglobin with 50 µmol/l NADPH at pH 7.5 (3);
methaemoglobin + 0.77 mmol/l postassium cyanide at pH 7.5 (4);
oxyhaemoglobin at pH 7.5 (5);
methaemoglobin + 0.77 mmol/l postassium cyanide at pH 7.5 (6);
50 µmol/l NADH (7) or NADPH (8) in buffer, pH 7.5.

Discussion

Bichromatic spectrophotometry is considerably more precise and sensitive than conventional monochromatic spectrophotometry. Furthermore, separate sample blanks are not necessary, even when test solutions are turbid (19). However, if a specific chromophore is present which displays variable absorb-

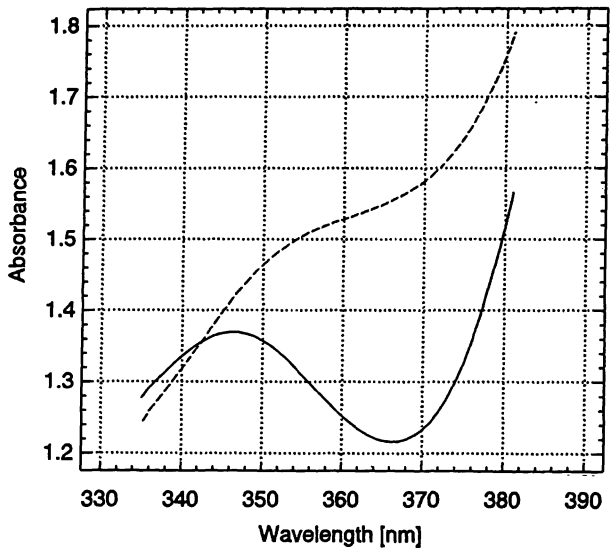

Fig. 9. Absorbance of 50 µmol/l haemoglobin in the 335–380 nm region at various oxygen saturations. sO_2 = 0.93 (——) or 0.35 (----). T = 25 °C, pH 7.5.

ance at the primary and secondary wavelength between sequential measurements, the reference reading will give an inadequate or even misleading correction. This type of spectral interference is observed with haemoglobin in the bichromatic spectrophotometry of NAD(P)H at 340/380 nm (or similar wavelengths) which is nowadays frequently applied in analytical procedures.

The oxidation of haemoglobin-O_2 with formation of Fe(III) derivatives resulting in a decrease of $\Delta A_{340/380\,nm}$ appears to be the most important cause of shifts of the sample blank absorbance in the UV range (figs. 2 and 5). The rate of methaemoglobin formation was 2.7-fold higher at 37 °C than at 25 °C. This explains the better stability of $\Delta A_{340/380\,nm}$ when using the "endpoint method" for measuring glucose in haemolysates at 25 °C (4, 5). The rate of methaemoglobin formation increases considerably below pH 7 (figs. 3, 4). pH-values < 7 are seldom applied in NAD(P)H-dependent methods but in the measurement of creatine kinase activity the buffer pH is 6.7 (20). Addition of plasma slowed the rate of haemoglobin autoxidation, resulting in better stability at pH values > 7 (fig. 4). Organic phosphates such as ATP or inositol hexakisphosphate (12) can positively influence the autoxidation rate (fig. 4). In the presence of 0.05 mmol/l NADH or NADPH, the decrease of $\Delta A_{340/380\,nm}$ was accelerated (fig. 4), even allowing for their spontaneous degradation (14) by using appropriate buffer blanks (fig. 8). It is not clear whether this was due to an effect on the haemoglobin oxidation, or to an increased rate of cosubstrate autoxidation in the presence of haemoglobin or other constituents of lysed cells.

As the drift of $\Delta A_{340/380\,nm}$ with methaemoblobin formation is predominantly caused by an increased absorbance at 380 nm (fig. 2), it may be necessary to use a reference wavelength beyond the *Soret* region (e.g. 475 nm, see fig. 2) for measurements with haemolysates at 37 °C. A reference wavelength near the peak absorbance of NAD(P)H, e.g. 374 nm (13) or a similar wavelength, is normally preferred, because the disturbing effects of *Raleigh* scatter are eliminated more effectively in this way.

The haemichrome formed in the presence of an excess of sodium lauryl sulphate (15) exhibits rather stable UV absorbance (fig. 6). Apart from possible chemical interference in enzymatic assays, sodium lauryl sulphate appears to be quite appropriate for the preparation of haemolysates. At lower concentrations, sodium lauryl sulphate caused a drift of $\Delta A_{340/380\,nm}$ as did the neutral detergent Triton X-100 (fig. 6). Digitonin, which is also used as a haemolysing agent (21), did not adversely affect the stability of haemoglobin.

Raising the temperature of haemoglobin-O_2 solutions caused reversible shifts of the absorbance between 340 and 380 nm. The observation of this process was impeded by the marked and simultaneous formation of methaemoglobin (fig. 7). The reversibility of the temperature-induced shift was shown by the inverse change of $\Delta A_{340/380\,nm}$ when the temperature was changed from 37 to 25 °C (fig. 8). Similar changes of $\Delta A_{340/380\,nm}$ were observed with a solution of cyanomethaemoglobin (fig. 8), which is chemically more stable. It is therefore improbable that a temperature-dependent dissociation of the haemoglobin-O_2 complex (s. below) caused the spectral changes. Because of the temperature-induced shifts of the haemoglobin absorbance, the reading of the sample blank should be performed only after reaching temperature equilibrium. If reagent and sample are kept at room temperature, an initial reading of $\Delta A_{340/380\,nm}$ immediately after mixing of sample and reagent will lead to a negative bias, as observed in the measurement of glucose in haemolysates with the incubation temperature set at 37 °C (4, 5). Possible temperature effects in bichromatic measurement have been mentioned in the literature (19).

As the isosbestic point of deoxyhaemoglobin and oxyhaemoglobin is near 340 nm, NAD(P)H concentrations can be measured monochromatically irrespective of the actual pO_2 (22). However, between 360 and 380 nm the absorptivity of haemoglobin strongly depends on the actual O_2-saturation (fig. 9). It is therefore to be expected that $\Delta A_{340/380\,nm}$ will be considerably influenced by pO_2 changes. There is as yet no evidence that this effects is of practical relevance in analytical

chemistry but it should be kept in mind that the sO_2 of haemoglobin is also influenced by pH, temperature and allosteric effectors.

The interference phenomena investigated in this study are traceable to changes in the spectral absorbance of haemoglobin that are largely independent of the presence of NAD(P)H. As the shifts merely affect the UV region where the reference readings are usually taken (370–380 nm) it is understandable that the spectral interference by haemoglobin was detected only by a thorough evaluation of a bichromatic measurement procedure (4, 5). It should be emphasized that the spectral interference by haemoglobin causes constant bias independent of the actual analyte concentration. Medically important bias may occur at haemoglobin concentrations as low as 20 μmol/l in the test solution as applied in routinely used procedures, e. g. in the measurement of glucose in blood (4). The interference previously observed in monochromatic measurement at much higher haemoglobin concentration (143 μmol/l) (3) and not below 62 μmol/l (23), produces a proportional bias, because the absorbance of reduced cosubstrate appears to be suppressed. An interaction between haemoglobin and the cosubstrate has been suggested (23, 24).

Acknowledgement

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