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SHORT COMMUNICATION/KURZMITTEILUNG

Stability Constants of Haemiglobin Cyanide and Azide Measured by Two-Wavelength Spectrophotometric Method

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Summary: A two-wavelength spectrophotometric procedure for the simultaneous determination of haemiglobin and haemiglobin cyanide (HiCN) (or of haemiglobin and haemiglobin azide (HiN_3)) concentrations in mixtures has been developed and applied to the determination of the stability constants of HiCN and HiN_3. The analytically reliable procedure allowed stability constants to be estimated with about 10% (relative standard deviation, coefficient of variation) uncertainty. Values of $1.9 \cdot 10^6$ and $2.0 \cdot 10^5$ $l \cdot mol^{-1}$ were obtained for HiCN and HiN_3, respectively, These results are discussed in relation to the optimal composition of the reagents for blood haemoglobin assay by the two methods.

Introduction

The haemiglobin cyanide (HiCN) method is a well established procedure for total haemoglobin measurement in blood (1). The method includes the conversion of haemiglobin (Hi) into HiCN; in an alternative method (2), Hi is converted into haemiglobin azide (HiN_3).

The molar absorption coefficient and the stability constant of the HiCN complex have recently been redetermined by titration with cyanide (3). We developed a two-wavelength spectrophotometric method for the simultaneous determination of Hi and HiCN (or of Hi and HiN_3) in mixtures, and applied it to the titrimetric measurement of the stability constants of HiCN and HiN_3.

Materials and Methods

Washed erythrocytes (from healthy non-smokers) were haemolysed with 1 volume of water and 0.5 volumes of tetrachloromethane. The haemolysates were supplemented with potassium hexacyanoferrate(III) (1.2 molar excess over Hb concentration), and dialysed overnight against potassium hexacyanoferrate solution, in order to achieve complete conversion. The Hi concentration, measured as HiCN (4), ranged from 3.4 to 6.9 mmol/l in the several preparations. Aliquots (50 µl) of the Hi solutions were mixed with 5000 µl of phosphate buffer (0.1 mol/l, pH = 7.1) and with 50 µl each of potassium cyanide solution (102 mmol/l), sodium azide solution (5.1 mol/l) and water, for the determination of absorptivities. For the titration experiments, the same dilution scheme was followed, using sets of KCN and NaN_3 solutions, with concentrations in the range 0–10 and 0–20 mmol/l, respectively. After 30 min conversion time, absorbance values were measured at 504 and 540 nm.

Volumes were measured with accurate (verified) dilutors and positive displacement pipettes; spectrophotometric measurements were made with a Varian DMS 90 instrument, whose absorbance and wavelength accuracy were checked with filters (NBS-SRM 930D) and with holmium perchlorate solution.

All calculations were referred to 1100 m^2/mol ($11.01 \cdot mmol^{-1} \cdot cm^{-1}$) as the absorption coefficient of HiCN at 540 nm, and to 16114.5 as the relative molecular mass of the haemoglobin monomer (1).

Results and Discussion

The measured absorption coefficients are shown in table 1. These values were introduced into two pairs of multicomponent-analysis equations, which were solved to yield Hi and HiCN (or Hi and HiN_3) concentrations, from the measured absorbance values at 504 and 540 nm. The calculated concentration values, plotted against the concentrations of KCN or

Tab. 1. Molar absorption coefficients of haemoglobin derivatives (mean values ± standard deviation, number of determination in brackets) in phosphate buffer, pH 7.1. Unit: m^2/mol.

	Hi	HiCN	HiN_3
504 nm	875 ±5 (24)	691 ±4 (26)	706 ±6 (13)
540 nm	647 ±8 (23)	1100	1123 ±5 (12)

NaN_3, are shown in figures 1 and 2. As usual, these plots show two linear parts: equations of these lines were calculated by means of linear regression ($r > 0.999$ in several experiments) and the abscissa value corresponding to the intersection of the lines (equivalence point) was calculated. From each experiment, two such values were calculated, one from the descending curve (disappearance of Hi) and one from the ascending curve (formation of HiCN or HiN_3): they agreed within ± 1%.

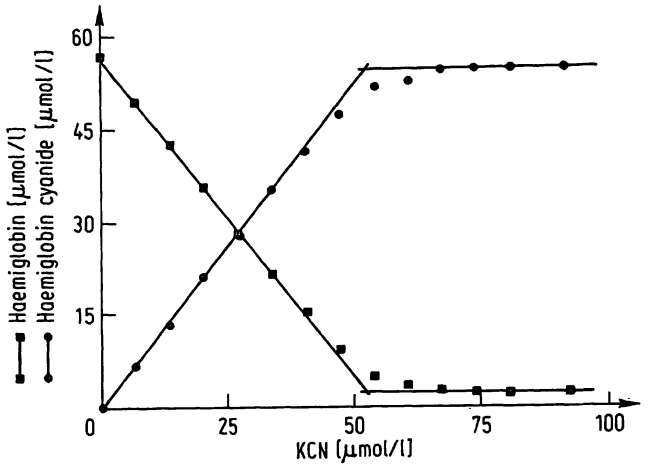

Fig. 1. Disappearance of haemiglobin (■) and formation of haemiglobin cyanide (●) with increasing concentrations of KCN.

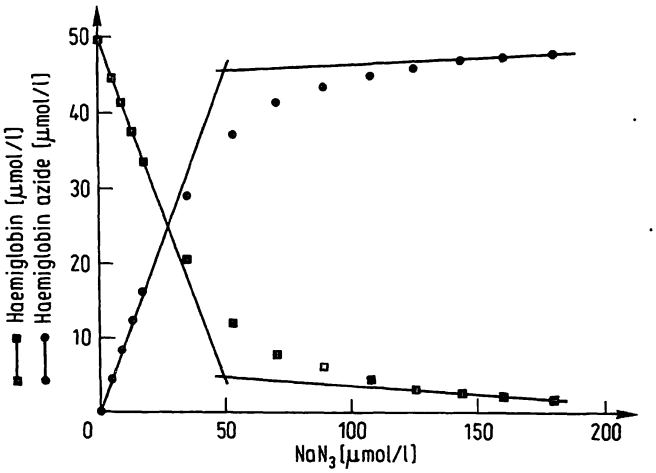

Fig. 2. Disappearance of haemiglobin (□) and formation of haemiglobin azide (●) with increasing concentrations of NaN_3.

Intermediate points were fitted by second degree polynomials, from which the concentrations of Hi and HiCN (or HiN_3) at the equivalence point were calculated.

From these concentrations, the degree of dissociation of the HiCN and HiN_3 complexes were calculated as (3):

$$\alpha_{HiCN} = \frac{[Hi]}{[Hi] + [HiCN]}; \quad \alpha_{HiN_3} = \frac{[Hi]}{[Hi] + [HiN_3]}$$

Stability constants for both complexes were calculated as (3):

$$K = \frac{1 - \alpha}{\alpha^2[Hb]},$$

where

$$[Hb] = [Hi] + [HiCN]$$

Results were (mean ± standard deviation, number of experiments in brackets):

$$K_{HiCN} = (1.89 \pm 0.27) \times 10^6\ l \cdot mol^{-1} \quad (n = 7)$$

$$K_{HiN_3} = (2.00 \pm 0.19) \times 10^5\ l \cdot mol^{-1} \quad (n = 5)$$

Correspondent pK′ values were:

$$pK'_{HiCN} = 6.28$$

$$pK'_{HiN_3} = 5.30$$

The present results confirm the higher stability of the HiCN complex (5), our values being in good agreement with previously reported ones (3, 5). As compared with a different experimental approach (3), our method allows more a precise determination, as judged from the standard deviation. Indeed, the two-wavelength method was found to give precise and accurate results in the course of a preliminary evaluation, using mixtures of known composition.

According to the measured stability constant of the HiCN complex, a concentration of about 0.53 mmol/l of CN^- is high enough to ensure 99.9% conversion of Hi into HiCN; as already observed (3), the KCN concentration (0.77 mmol/l) in the recommended reagent (4) is therefore adequate. On the other hand, the NaN_3 concentration of 0.46 mmol/l in the recommended reagent (2) allows only about 99% conversion of Hi into HiN_3: the NaN_3 concentration in the reagent should therefore be raised to about 5.0 mmol/l for adequate conversion. Incomplete conversion could be the reason for some discordant reported values (6) for the ratio $\varepsilon_{HiN_3}/\varepsilon_{HiCN}$ at 542 nm. From present results (tab. 1), the value for this ratio, at 540 nm, is 1.02.

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