To the Editor: In a recent publication in this Journal Zijlstra, Buursma, and Zwart (5) cast doubts on our results (2) concerning the influence of the concentration of hemoglobin (Hb) on the visible spectrum of Hb. In this letter we want to discuss critically the experiments and conclusions of Zijlstra et al. and to present our point of view.

First, Zijlstra et al. stated that their results may invalidate the cyanmethemoglobin method, which is generally used for the determination of content of hemoglobin in blood (cHb). Conversely, we believe that different Hb species also show a different association behavior. Cyanmethemoglobin, especially under the conditions applied in the hemoglobin cyanide (HiCN) method, may not associate. Furthermore, in general, a relatively small range of cHb is covered in the application of the HiCN method; therefore any influence of cHb is likely to be unimportant.

Moreover, Zijlstra et al. (5) stated that they are unable to disprove our results with their experiments. However, they used other experimental conditions, such as different ionic composition of the solution and temperature. The Cl− concentrations in both experiments differed considerably; in addition, Zijlstra et al. used tris(hydroxymethyl)methylamine (Tris) ions as counter ions. The latter seems to be an essential difference, since Tris ions act as a buffer system and are therefore not inert. In addition, Zijlstra et al. made their measurements at room temperature, whereas our measurements were made at 37°C. It was not our intent to use an artificial system and artificial conditions. Instead, we attempted to determine the properties of Hb molecules in a physiological environment. Further studies are needed to clarify whether the optical properties of Hb solutions are changed remarkably by Tris ions. Until then, care must be taken in comparing our results with those of Zijlstra et al.

In our opinion, the association behavior of different Hb derivatives depends strongly on the ionic composition of the solution. In a similar way 2,3-diphosphoglycerate (2,3 DPG) binding to Hb may be influenced by different experimental conditions. Zijlstra et al. (5) have shown that 2,3-DPG does not influence the visible spectrum of Hb under chosen conditions. In contrast, Adams and Schuster (1) have demonstrated a clear influence of organic phosphates, including 2,3-DPG, on the visible spectrum of Hb under different experimental conditions.

Furthermore, we have pointed out that most publications concerning Hb spectra do not contain clear descriptions of the experimental conditions. In particular, the Hb concentration is often overlooked. Even though Zijlstra et al. cite some publications to suggest that this problem has been extensively considered in the literature, we know of only two publications (3, 4) that clearly give Hb concentrations. Drabkin (3) measured Hb solutions with a cHb of 8 mmol·(Fe−)·l−1 and reported an extinction coefficient (εHbO2) of 15.341 mmol·Fe−1·cm−1 (λ = 578 nm). Van Assendelft used a cHb of about 5 mmol·Fe−1·l−1 and found an εHbO2 of 15.37 l·mmol·Fe−1·cm−1 at the same wavelength. In both publications some important experimental conditions are not given. For example, it is not known what kind of solution was used to adjust cHb. According to our results, εHb in this range of cHb is 18.01 l·mmol−1·Hb−1·cm−1 (λ = 575 nm). Perhaps this discrepancy is caused by different composition of the solutions used. Further consideration is also warranted whether hemolysis by Sterox SE (5) causes additional effects.

Finally, we want to emphasize that Zijlstra et al. have measured the visible Hb spectrum only in the high λ range. Especially in the Soret region we have observed changes in εHbO2 as well as spectral shifts. To summarize, we do not have any doubt concerning the validity of the HiCN method. We also believe that direct comparison between the results in both publications is impossible because the experimental conditions differ markedly. The experiments of Zijlstra et al. (5) did not disprove our results. To compare results, they would have had to repeat our experiments using identical experimental conditions.

REFERENCES
To the Editor: It is encouraging that Burkhard and Barnikol (1) now clearly express their confidence in the hemoglobin-cyanide method for the determination of total hemoglobin (cHb) in blood. In their previous paper (2) the reader could hardly escape the conclusion that the authors were very much in doubt about the validity of this method, since they made their cHb measurements at the same final concentration as used in the other measurements, with the stated purpose “to rule out the influence of the protein concentration on the spectra of cyanmethemoglobin (which may exist) falsifies [the] results.”

In the summary of their letter Burkhard and Barnikol state that we should have exactly repeated their experiments to disprove their results. This would have been a fair demand if the authors had tried to demonstrate that under certain special conditions the absorptivity of hemoglobin depends on cHb. However, their claim is much more general, namely that Lambert-Beer’s law simply does not hold for HbO2, and it is this general claim that we wanted to disprove. We did not want to disprove just their limited experimental results, nor did we want to enter into the discussion of the possible aggregation of hemoglobin tetramers with which the authors seem so preoccupied. It was our purpose to show, with evidence from the literature and from our own laboratory, that for all practical purposes Lambert-Beer’s law is valid for solutions of human hemoglobin so that the concentration of hemoglobin and its common derivatives can be accurately measured by spectrophotometry.

In our opinion there is convincing evidence that the medium in which the measurements are made has little or no influence on the absorption spectra of hemoglobin. The factors mentioned by Burkhard and Barnikol in their letter have for the most part already been discussed in our paper (6). Briefly, the indifference of Cl- concentration (cCl-) and the use of tris(hydroxymethyl)methylamine (Tris) buffer follows from a comparison of the data of Fig. 1 (Tris buffer with cCl = 50 mmol l-1) with those of Fig. 2 (Locke’s solutions with cCl = 159 mmol l-1); the absorption spectra in these media are identical. The factors temperature and hemolysis by Sterox SE are covered by a comparison of data from Van Assendelft (5) and Siggaard-Andersen et al. (4). Van Assendelft, measuring at room temperature after Sterox hemolysis with cHb ≈ 5 mmol l-1, finds 11.27 mmol l-1 cm-1 for cHbO2 at 559 nm, whereas Siggaard-Andersen et al. (4), measuring at 37°C after hemolysis by freezing and thawing, find 11.29 mmol l-1 cm-1 at 570 nm for an erythrolysate with cHb ≈ 20 mmol l-1. A striking example of the indifference of the medium and the total concentration can also be found in Drabkin’s 1946 paper (3), cHbO2 at 578 nm was measured for two solutions made from crystallized human hemoglobin. For a solution in concentrated (NH4)2SO4 with cHb = 38.2 mmol l-1, cHbO2 was 15.41 mmol l-1 cm-1, whereas in a salt-free dialysate with cHb = 0.1083 mmol l-1, a value of 15.43 mmol l-1 cm-1 was found. It is evidently not true that most authors reporting absorptivity data of hemoglobin do not specify their measuring conditions and cHb.

The data in Table 2 of our paper (6) are indeed the result of independent measurements. The spread in these data is not incredibly low. This may be demonstrated by the results of two new series of measurements, of which we quote only the results of HbO2 at λ = 542 and 540 nm: 14.60 ± 0.09 and 14.44 ± 0.09 mmol l-1 cm-1 (n = 46; N = 17) for a Sterox hemolysate of whole blood and 14.44 ± 0.08 and 14.27 ± 0.08 mmol l-1 cm-1 (n = 20; N = 6) for a toluene erythrolysate of washed red blood cells (n, no. of independent measurements; N, no. of donors).

In Table 1, the mean absorptivity values are compared with some earlier data, showing a fair agreement in spite of the quite different ways of preparing the measuring solutions. The whole blood values are slightly higher due to some unavoidable turbidity. We still do not understand how Burkhard and Barnikol could arrive at cHbO2 = 19.3 mmol l-1 cm-1 (2).

REFERENCES