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Spectrophotometry of Hemoglobin: Absorption Spectra of Bovine Oxyhemoglobin, Deoxyhemoglobin, Carboxyhemoglobin, and Methemoglobin

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ABSTRACT. The absorptivity at 540 nm of bovine hemiglobincyanide (cyanmethemoglobin) was determined on the basis of the iron content and found to be equal to the established value for human hemiglobincyanide (11.0 L · mmol^{-1} · cm^{-1}). On this basis the absorption spectra of the common derivatives were determined for bovine hemoglobin. There proved to be only slight differences in the oxyhemoglobin, deoxyhemoglobin, and carboxyhemoglobin spectra between bovine and human hemoglobin. For comparison of the methemoglobin spectra a new series of measurements was made for human hemoglobin. As also found in the rat, the methemoglobin spectrum of bovine blood differed considerably from that in the human. These differences should be taken into account in multicomponent analysis. COMP BIOCHEM PHYSIOL 118B;4:743–749, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Bos tauris, hemoglobin, hemoglobinometry, hemiglobin, absorption spectra, carboxyhemoglobin, methemoglobin, cyanmethemoglobin

INTRODUCTION

Because the visual spectrum of hemoglobin is essentially determined by the iron-porphyrin moiety and because the spectral differences between the common hemoglobin derivatives are due to differences in ligand binding to the iron, and/or to a change from the ferrous to the ferric state, it has long been assumed that no difference in the spectral properties of their hemoglobin would be present between various mammals and even birds. However, the finding of slight, but analytically relevant spectral differences between human fetal and adult hemoglobin (9,13) raised the question if similar, or even greater, differences would occur between different animals. For obvious reasons this would be especially important in the case of laboratory animals, whose blood is often examined by spectrophotometric methods designed for human blood. Therefore, we have determined the absorption spectra of the common derivatives of dog (*Canis familiaris*) and rat (*Rattus rattus*) hemoglobin. No significant differences were found between dog and human hemoglobin (11), but between rat and human hemoglobin there were differences, especially in the methemoglobin spectrum, which should certainly be taken into account in spectrophotometric multicomponent analysis of rat blood (12).

Recently, much attention has been paid to bovine blood as a substitute source of hemoglobin for the International Haemiglobincyanide Standard, which, up to the present, has been made exclusively from human hemoglobin (3), and for quality control material for multiwavelength hemoglobin photometers ("CO-oximeters") (5). Bovine blood is more readily available in large quantities, and its use in the production of quality control materials obviates the need for elaborate procedures to avoid contamination with pathogenic human viruses, such as human immunodeficiency virus (HIV) and hepatitis virus.

For bovine blood to be a suitable starting material for the International Haemiglobincyanide Standard and related reference materials for hemoglobinometry, it had to be ascertained that the absorptivity at 540 nm (ε^{540}) of bovine hemiglobincyanide (HiCN; also called cyanmethemoglobin) is not significantly different from the human value. For human blood, $\varepsilon^{540}{}_{HiCN}$ has been determined on the basis of iron, nitrogen and carbon (7) and a value of 11.0 L · mmol^{-1} · cm^{-1} has been established as the basis of the reference method for hemoglobinometry (2,3). Therefore, and because $\varepsilon^{540}{}_{HiCN}$ is the anchor value on which all absorptivity spectra of hemoglobin derivatives are based, we started the investigation of the spectrophotometric properties of bovine blood with a determination of $\varepsilon^{540}{}_{HiCN}$. Subsequently we determined the spectra of oxyhemoglobin, deoxyhemo-

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TABLE 1. Millimolar absorptivity of HiCN at λ = 540 nm for bovine blood

No.	ε^{540}_{HiCN}	*N*
1	10.91	5
2	10.93	5
3	10.99	6
4	10.90	8
5	10.97	5
6	10.91	5
7	10.97	5
8	11.00	4
9	11.04	7
10	11.00	5
11	10.89	6
12	10.94	5
13	11.04	5
14	10.94	6
15	10.95	5
Mean	10.96	10.96
SD	0.05	0.07
SEM	0.01	0.01
N	15	
n		82

ε^{540}_{HiCN} is expressed in L · mmol^{-1} · cm^{-1}. Standard deviation (SD) and standard error of the mean (SEM) have been calculated for the mean values of the stock solutions (*N*) and for the individual measurements (*n*).

globin, carboxyhemoglobin, and methemoglobin (hemiglobin), as earlier carried out for human, dog, and rat blood (7,11–13).

MATERIALS AND METHODS

Blood from healthy cows (*Bos taurus*) was obtained at a butcher and collected in iron-free flasks containing 100 USP units of sodium heparin per ml of blood. Since bovine hemoglobin is well-soluble in water, clear stroma-free stock solutions with a concentration of c. 8 mmol/l (129 g/l, the relative mass of the bovine hemoglobin monomer being 16133) can easily be prepared by the simple method as described for dog blood (11). Briefly, after washing the red cells three times with isotonic saline solution, 1 vol of erythrocytes is thoroughly mixed with 1 vol of distilled water and 0.4 vol of toluene. After storing at 4°C for at least 16 hr, the mixture has separated into three layers, of which the lowest is a clear stroma-free hemoglobin solution. This solution is centrifuged for 20 min at 8000 ***g***, filtered through ash-free paper, and stored at 4°C.

The iron concentration of the hemoglobin stock solutions was determined with the α,α'-dipyridyl method (11,15). In brief, 1 ml of hemoglobin solution was decomposed by the addition of HNO_3, 65%, and H_2O_2, 30%, and slow heating to 220°C, until a clear, yellow solution was obtained, which then was evaporated almost to dryness. Excess acid was removed by the addition of water and evaporation *in vacuo* at 95°C. The solution was transferred to a volumetric flask and made up to 100 ml with water. Then 5 ml of this solution was mixed with 1 ml buffered α,α'-dipyridyl solution and 1 ml sodium sulfite solution, and heated to 95–100°C for 10 min. Thus, a pink solution was obtained, which, after cooling, was measured at 520 nm on an Optica CF4 grating spectrophotometer (Optica, S.p.A., Milan, Italy) with a lightpath length of 1.000 cm. The iron concentration was obtained with the help of a calibration curve made using ferric ammonium sulphate, $NH_4Fe(SO_4)_2 \cdot 12\ H_2O$, the iron content of which had been checked by gravimetric analysis.

For the determination of the absorbance (*A*) of HiCN at 540 nm, 1 ml of hemoglobin stock solution was made up to 250 ml with a solution containing 200 mg $K_3Fe(CN)_6$, 50 mg KCN and 1.0 g $NaHCO_3$ per liter. The absorbance at 540 nm of this solution was determined, after at least 20

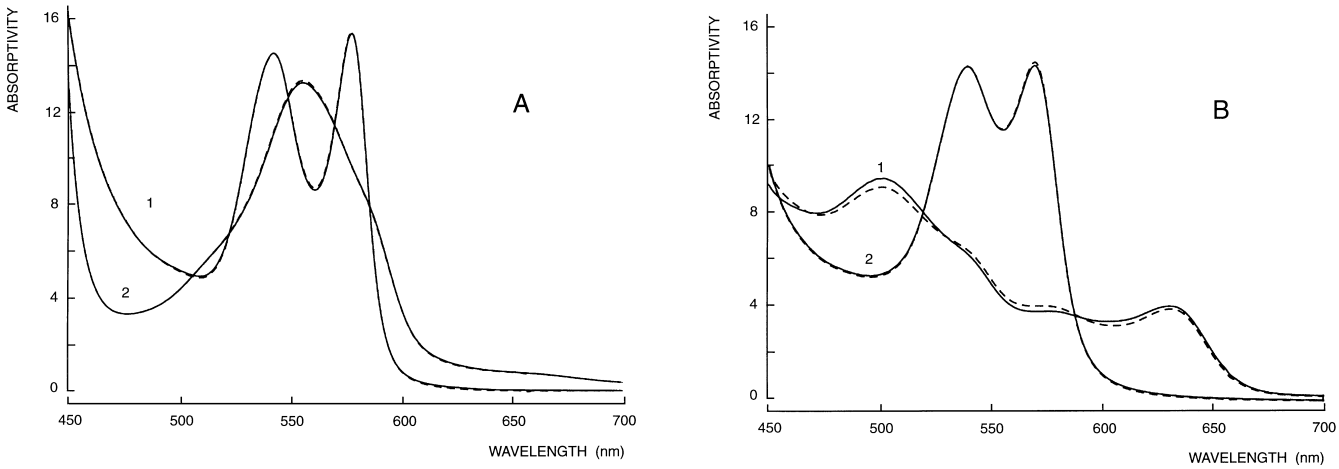

FIG. 1. Absorption spectra of the common derivatives of bovine (—) and human (– – – –) hemoglobin in the visible range. (A) oxyhemoglobin (1) and deoxyhemoglobin (2); (B) methemoglobin (1) and carboxyhemoglobin (2). The absorptivity is expressed in L · mmol^{-1} · cm^{-1}.

TABLE 2. Millimolar absorptivities near principal peaks and troughs in the spectra of HHb, O_2Hb, COHb, and Hi for bovine and human blood

		Bovine		Human		
Hb species Min/Max	**λ (nm)**	**ε^{λ} ± SEM**	***N***	**ε^{λ} ± SEM**	***N***	***P***
HHb						
Min	476	3.32 ± 0.010	10	3.33 ± 0.019	6	—
Max	554	13.25 ± 0.031	10	13.34 ± 0.052	6	—
O_2HB						
Min	508	4.94 ± 0.009	8	4.88 ± 0.008	6	<0.001
Max	540	14.35 ± 0.027	8	14.32 ± 0.030	6	—
Max	542	14.53 ± 0.032	8	14.52 ± 0.025	6	—
Min	560	8.68 ± 0.012	8	8.77 ± 0.017	6	<0.001
Max	576	15.19 ± 0.034	8	15.26 ± 0.037	6	—
Max	578	15.33 ± 0.034	8	15.36 ± 0.030	6	—
COHb						
Min	496	5.28 ± 0.010	8	5.22 ± 0.018	8	<0.02
Max	538	14.27 ± 0.025	8	14.30 ± 0.041	8	—
Max	540	14.26 ± 0.031	8	14.27 ± 0.038	8	—
Min	554	11.60 ± 0.022	8	11.63 ± 0.031	8	—
Max	568	14.29 ± 0.027	8	14.43 ± 0.040	8	<0.01
Max	570	14.31 ± 0.022	8	14.46 ± 0.042	8	<0.01
Hi						
Max	500	9.48 ± 0.024	8	9.11 ± 0.029	15	<<0.001
Max	632	4.02 ± 0.016	8	3.90 ± 0.021	15	<0.001

ε^{λ} is expressed in L · mmol^{-1} · cm^{-1}; *N* is number of animals (humans); only *P* values <0.05 are given.

min, on an Optica CF4 spectrophotometer with a lightpath length (l) of 1.000 cm. The millimolar absorptivity ($\epsilon^{540}{}_{HiCN}$), referring to one heme group and one globin moiety, was calculated using the equation:

$$\varepsilon^{540}{}_{HiCN} = A^{540}/c_{HiCN} \cdot l \qquad (1)$$

where c_{HiCN} is the HiCN concentration in mmol/l as determined on the basis of the iron concentration of the stock solution.

Clear solutions of oxyhemoglobin (O_2Hb), deoxyhemoglobin (HHb), carboxyhemoglobin (COHb), and methemoglobin (hemiglobin; Hi) were prepared as described elsewhere for human hemoglobin (13). From fresh bovine blood, anticoagulated with sodium heparin (100 USP units per ml of blood), the plasma was removed and the erythrocytes were resuspended in 9 g/l (0.154 mol/l) saline solution. The total hemoglobin concentration was kept between 100 and 150 g/l (6.2–9.3 mmol/l). O_2Hb, HHb, and COHb solutions were prepared by tonometry of *c.* 20 ml erythrocyte suspension in a cylindrical glass tonometer with O_2/CO_2, N_2/CO_2, and $N_2/CO/CO_2$ mixtures, respectively. After 2 hr of tonometry, erythrolysis was brought about

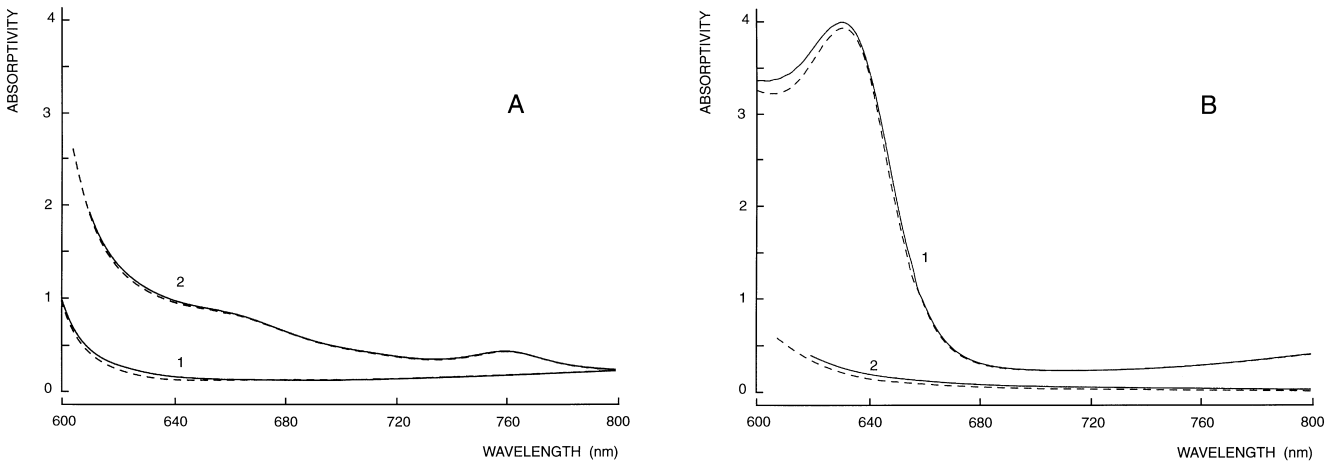

FIG. 2. Absorption spectra of the common derivatives of bovine (—) and human (– – – –) hemoglobin in the red and near-infrared range. (A) oxyhemoglobin (1) and deoxyhemoglobin (2); (B) methemoglobin (1) and carboxyhemoglobin (2). The absorptivity is expressed in L · mmol^{-1} · cm^{-1}.

through the introduction of 2 ml of a 100 ml/l solution of the nonionic detergent Sterox SE (Hartman, Leddon Co., Philadelphia, PA), into the revolving tonometer; this solution was in equilibrium with the gas mixture flowing through the tonometer. After erythrolysis, tonometry was continued for about 20 min. Hi was prepared from O_2Hb by the addition of 1.5 times the equimolar amount of solid $K_3Fe(CN)_6$ to a O_2Hb solution of *c*. 5 mmol/l (80.7 g/l). After 15 min the solution was transferred to a glass syringe.

For O_2Hb and COHb, the spectrophotometer cuvets were filled directly from the tonometers through cotton wool filters; contact between the sample to be measured and room air was avoided. For HHb, the cuvets were filled by a slightly different procedure, allowing the addition of 3 mg of sodium dithionite ($Na_2S_2O_4$) per 2 ml of erythrolysate. For Hi, the cuvets were filled directly from the glass syringe through cotton wool filters.

Absorbance measurements were made at room temperature (20–24°C) with a Model HP8450A diode-array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) in the spectral range of 450–700 nm with $l = 0.013$ cm, and in the range of 600–800 nm with $l = 1.000$ cm for COHb, with $l = 0.203$ cm for O_2Hb and HHb, and with $l = 0.100$ cm for Hi. The samples used for the measurements in the latter range were subsequently measured in the range of 600–1000 nm at various intervals with an Optica CF4 spectrophotometer, at the same values for lightpath length. In each sample, the total hemoglobin concentration (*c*tHb) was determined by means of the standardised hemiglobincyanide method (2,3). The pH of the Hi containing samples was determined with a Model PHM28 pH meter (Radiometer A/S, Copenhagen, Denmark).

The absorptivities of the hemoglobin derivatives at each wavelength (ε^{λ}, expressed in $L \cdot mmol^{-1} \cdot cm^{-1}$) were calculated, analogous to Equation (1), by dividing the absorbances by *c*tHb (in mmol/l) and l (in cm). For Hi, this gives correct values for ε^{λ}. For O_2Hb, HHb, and COHb, the measured values of ε^{λ} must be corrected for any contaminating Hi. To this end, the Hi fraction (F_{Hi}) of each sample was determined with a Hemoximeter OSM3 (Radiometer) (14), and the absorptivity of derivative x was calculated with the equation:

$$\varepsilon^{\lambda}_{x} = (\varepsilon^{\lambda} - F_{Hi} \cdot \varepsilon^{\lambda}_{Hi})/(1 - F_{Hi}) \qquad (2)$$

where $\varepsilon^{\lambda}_{x}$ is the corrected absorptivity of O_2Hb, HHb, and COHb at wavelength λ, $\varepsilon^{\lambda}_{Hi}$ is the absorptivity of Hi, and ε^{λ} is the measured absorptivity of O_2Hb, HHb, and COHb.

For comparison, the absorptivities of the corresponding derivatives of human hemoglobin from (13) were used, with the exception of the values for methemoglobin. The absorptivities of this derivative have been recalculated using both earlier recorded spectra and some newly recorded ones of fresh human blood, using the technique described above. The revised data for human Hi as measured with the HP8450A are now based on the blood of 15 healthy humans, and the data obtained with the Optical CF4 are based on seven specimens of human blood.

The statistical significance of the differences between the absorptivities of the hemoglobin derivatives of bovine and human blood was assessed by Student's *t*-test for unpaired samples, two-tailed. A difference was considered significant when P was < 0.05.

RESULTS

ε^{540}_{HiCN} was determined in 15 stock solutions. Of each stock solution, 4–8 separate determinations were made, giving a total of 82 independent measurements. The results are shown in Table 1.

Figure 1 shows the absorption spectra of the four hemoglobin derivatives from 450–700 nm for bovine hemoglobin as determined with the HP8450A spectrophotometer, in comparison with the corresponding spectra of adult human hemoglobin. The absorptivities of the various hemoglobin derivatives at wavelengths near the principal light absorption maxima and minima are presented in Table 2. The data show that most of the slight differences discernible in the graphs of Fig. 1 are statistically significant.

Figure 2 shows the bovine and human absorption spectra of the various derivatives in the spectral range of 600–800 nm; an expanded ordinate is used because the absorptivities are lower in this region. There are no appreciable differences in the absorption spectra of HHb, O_2Hb, and COHb between bovine and human hemoglobin in this range. For Hi, panel B shows that at $\lambda > 640$ nm there is no appreciable difference anymore between the absorptivity spectra of bovine and human hemoglobin.

Table 3 shows a selection of absorptivities of the four derivatives of bovine and human hemoglobin through the entire range of 450–1000 nm, including some wavelengths of interest for special purposes, *e.g.*, 660 and 940 nm, which are used in pulse oximetry, and 700, 775, 805, 845, 880, and 904 nm, which are used in near-infrared spectrophotometry of organs and tissues *in vivo* (1). The values for 450–600 nm are based on measurements with the HP8450A spectrophotometer, those for 600–1000 nm on measurements with the Optica CF4. For comparison, the corresponding data for adult human hemoglobin have been added.

In the case of methemoglobin, a comparison between the absorptivity spectra of different species can only be properly made when the influence of any differences in pH are taken into account. For the data of Figs. 1 and 2, and of Tables 2 and 3, the average pH value of the measured methemoglobin solutions was 7.28 and 7.21 for bovine and human hemoglobin, respectively. As shown in Table 2, at the maxima near 500 and 632 nm, the absorptivity of bovine Hi is significantly greater than that of human Hi. In part of the spectral region between the two maxima, the position of the absorptivity curves has reversed: $\varepsilon^{570}_{Hi} = 3.77 \pm 0.020$ and 4.01 ± 0.024 $L \cdot mmol^{-1} \cdot cm^{-1}$, for bovine and human

TABLE 3. Millimolar absorptivities of four derivatives of bovine (B) and human (H) hemoglobin at selected wavelengths in the visible and near-infrared spectral range

	ε_{HHb}		ε_{O_2Hb}		ε_{COHb}		ε_{Hi}	
λ (nm)	**B**	**H**	**B**	**H**	**B**	**H**	**B**	**H**
HP8450A								
	$N = 10$	$N = 6$	$N = 8$	$N = 6$	$N = 8$	$N = 8$	$N = 8$	$N = 15$
450	13.40	13.51	16.13	16.20	10.13	10.07	9.21	9.80
480	3.34	3.35	6.71	6.72	5.67	5.62	8.24	8.07
500	4.34	4.34	5.20	5.15	5.34	5.28	9.48	9.11
510	5.38	5.38	4.94	4.88	6.00	5.94	9.02	8.67
520	6.45	6.48	6.05	5.98	8.33	8.30	7.94	7.73
530	7.99	8.04	10.41	10.35	12.30	12.29	6.95	6.94
538	9.88	9.94	13.92	13.90	14.27	14.30	6.35	6.50
540	10.42	10.50	14.35	14.32	14.26	14.27	6.17	6.34
542	11.03	11.09	14.53	14.52	14.02	14.04	5.98	6.17
550	12.88	12.97	11.98	12.01	12.10	12.11	4.97	5.16
554	13.25	13.34	10.10	10.17	11.60	11.63	4.47	4.66
560	13.01	13.09	8.68	8.77	12.18	12.25	3.98	4.18
568	11.80	11.85	10.39	10.50	14.29	14.43	3.78	4.01
570	11.40	11.44	11.58	11.68	14.31	14.46	3.77	4.01
576	10.06	10.07	15.19	15.26	11.74	11.76	3.79	4.00
578	9.64	9.62	15.33	15.36	10.26	10.27	3.78	3.99
580	9.21	9.19	14.46	14.42	8.69	8.64	3.77	3.95
590	6.91	6.87	4.23	4.26	2.94	2.89	3.54	3.56
600	3.76	374	0.98	0.96	1.15	1.10	3.36	3.23
630	—	—	—	—	—	—	4.03	3.89
Optica CF4								
	$N = 8$	$N = 7$	$N = 8$	$N = 6$	$N = 8$	$N = 6$	$N = 7$	$N = 7$
630	1.08	1.06	0.17	0.11	0.22	0.19	—	—
660	0.81	0.81	0.10	0.08	0.09	0.06	0.86	0.82
680	0.60	0.61	0.09	0.09	0.05	0.03	0.26	0.26
700	0.44	0.44	0.10	0.09	0.04	0.02	0.20	0.20
750	0.39	0.39	0.14	0.14	0.03	0.01	0.25	0.25
775	0.30	0.29	0.17	0.17	0.03	0.01	0.32	0.32
800	0.21	0.20	0.21	0.20	0.02	0.01	0.40	0.40
805	0.21	0.20	0.21	0.21	0.02	0.01	0.42	0.415
840	0.19	0.19	0.25	0.25	0.02	0.01	0.53	0.52
845	0.19	0.19	0.26	0.25	0.02	0.01	0.55	0.53
880	0.21	0.20	0.29	0.28	0.02	0.01	0.63	0.61
904	0.22	0.21	0.30	0.30	0.02	0.00	0.68	0.66
920	0.21	0.21	0.31	0.30	0.02	0.00	0.72	0.70
940	0.19	0.18	0.31	0.29	0.01	0.00	0.75	0.73
960	0.14	0.14	0.30	0.28	0.01	0.00	0.78	0.75
1000	0.07	0.06	0.27	0.25	0.01	0.00	0.80	0.76

ε is expressed in L · mmol^{-1} · cm^{-1}; N is number of animals (humans). HHb = deoxyhemoglobin; O_2Hb = oxyhemoglobin; COHb = carboxyhemoglobin; Hi = methemoglobin (hemiglobin).

hemoglobin, respectively ($P < 0.001$). Figure 3 demonstrates that the difference between bovine and human methemoglobin spectra is definitely not caused by the difference in pH. There is no pH value at which the absorption spectrum of bovine methemoglobin completely coincides with the spectrum of human methemoglobin.

DISCUSSION

The value of 10.96 ± 0.01 L · mmol^{-1} · cm^{-1} obtained for bovine hemoglobin is in excellent agreement with the values of 10.93 ± 0.005, 10.96 ± 0.01, and 10.97 ± 0.03 L · mmol^{-1} · cm^{-1}, for human, dog, and rat hemoglobin, obtained earlier on the basis of the iron content of hemoglobin measured by means of the same procedure (11,12,15). These values are also well within the range of values of ε^{540}_{HiCN} underlying the internationally accepted value for human hemoglobin of 11.0 L · mmol^{-1} · cm^{-1} (2,3). This value of ε^{540}_{HiCN} has been chosen on the ground of the results of 14 independent series of measurements based on either the iron, nitrogen, or carbon content of adult human hemoglobin [*cf.* Table 1 in (7)]. When one takes from these 14 investigations the three large series of determinations of ε^{540}_{HiCN} on the basis of iron determined with the α,α'-dipyridyl method, a value of 10.97 ± 0.01 L · mmol^{-1} · cm^{-1} (SEM; $N = 204$) is obtained. Hence, there is no reason to depart,

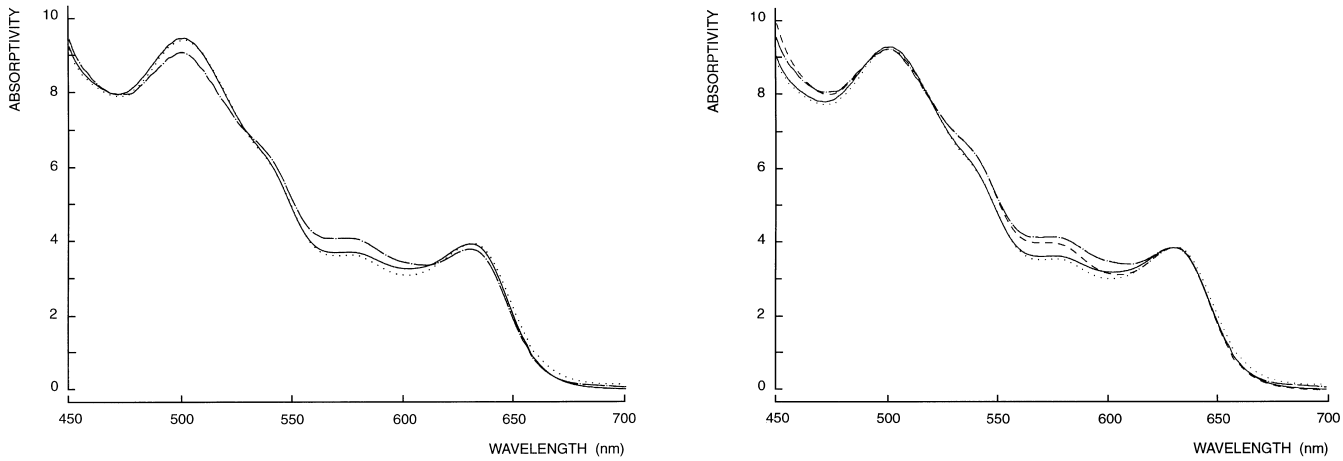

FIG. 3. Absorption spectra of bovine and human hemoglobin at various pH values. In the left panel the absorptivity spectra of bovine Hi are shown at pH 6.95 (·····), 7.28 (—) and 7.51 (–·–·–·–). In the right panel the same absorptivity spectra are shown, but with the absorptivity at λ = 630 nm normalized to 4.025 L · mmol^{-1} · cm^{-1}. An absorptivity curve of human Hi at pH 7.21 has been added (––––).

for bovine hemoglobin, from the rule that all absorptivities of hemoglobin derivatives are calculated on the basis of $\varepsilon^{540}{}_{HiCN}$ = 11.0 L · mmol^{-1} · cm^{-1}.

A fortunate consequence of the fact that $\varepsilon^{540}{}_{HiCN}$ of bovine hemoglobin does not deviate from the human value is that bovine blood can be used as a source for hemiglobincyanide standard solutions, as has already tentatively been applied in the production of a standard solution for hemoglobinometry issued by the *Bureau Communautaire de Recherche*, a central research organisation of the European Union.

The graphs presented in Fig. 1 show that for HHb, O_2Hb, and COHb there are only minute spectral differences between bovine and human hemoglobin, though some are statistically significant (Table 2). In the O_2Hb spectrum the minimum at 560 nm is slightly lower for bovine than for human hemoglobin, while at 508 nm the absorptivity of bovine hemoglobin slightly exceeds that of human hemoglobin. In the COHb spectrum the α-peak around 569 nm is somewhat lower for bovine hemoglobin, but in the minimum near 496 nm the spectrum of bovine hemoglobin lies a little above that of the human. The maximum in the bovine HHb spectrum at 554 nm seems to be somewhat lower than that in the spectrum of human hemoglobin (Fig. 1, panel A), but this difference did not reach statistical significance. Although these differences are but slight, they may be relevant in multicomponent analysis, either carried out by a general purpose instrument as the HP8450A or by a multiwavelength hemoglobin photometer ("CO-oximeter").

As shown in Fig. 2 and Table 3, there are, in the red and near-infrared region, no spectral differences between bovine and human hemoglobin as far as HHb, O_2Hb, and COHb are concerned. In panel A of Fig. 2 all four spectra converge to the isosbestic point, which is found almost exactly at 800 nm. Formerly, this isosbestic point was often found at a considerably higher wavelength. This, however, has been proven to be caused by a side reaction of the use of sodium dithionite for the deoxygenation of O_2Hb in the preparation of HHb. When HHb is prepared by tonometry and only a minute amount of dithionite used to prevent reoxygenation, the isosbestic point is found at its proper place (6,13).

Comparison of the Hi spectra of different kinds of hemoglobin is complicated by the fact that these spectra are, to a considerable extent, dependent on the pH of the solution. For practical reasons the pH of choice for the hemoglobin spectra is the mean value of the pH, which ensues when blood from healthy individuals is hemolysed. In fact, this is what happens in multicomponent analysis (10,14). Therefore, the standard Hi spectrum of bovine hemoglobin has been recorded at pH 7.28, while that of human methemoglobin has been determined at 7.21. Table 4 shows that at

TABLE 4. Methemoglobin fractions as determined by MCA for a pure bovine methemoglobin solution at various pH values

pH	F_{Hi} (%)
6.83	102.5
6.95	101.4
7.18	100.7
7.28	100.0
7.38	97.4
7.51	94.9
7.70	90.4

MCA is multicomponent analysis with an HP8450A reversed optics spectrophotometer in the system HHb/O_2Hb/COHb/Hi. The standard Hi spectrum used in the calculation was recorded at pH 7.28.

pH 7.28 multicomponent (HHb, O_2Hb, COHb, Hi) analysis applied to a bovine blood sample containing only Hi yields 100% indeed. At a lower pH a small overestimation occurs, but at higher pH values the measured Hi fraction is clearly too low.

The spectral change due to differences in pH is caused by binding of an OH^- ion to Hi. The difference between the Hi spectra of dog and human hemoglobin at the same pH proved to be caused solely by a different affinity of dog and human Hi for OH^- (11). Figure 3, however, shows that there is no pH at which the Hi spectra of bovine and human hemoglobin can be made to coincide. A similar result was found in the comparison of rat and human methemoglobin spectra (12). Hence, in these species there is a real difference between the Hi spectra.

The absorption spectrum of HHb is not dependent on the pH of the solution (8). For O_2Hb there is a pH effect indeed (8), but the effect is so small that, for the pH variations that occur in preparing the O_2Hb solutions, it is within the measuring error. The effect of pH variations on the COHb spectrum is still less (4).

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