SIMULTANEOUS DETERMINATION OF HEMOGLOBIN AND CYSTEINE BASED ON BRDIČKA CATALYTIC CURRENTS

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Dedicated to the memory of Prof. J. Heyrovský on the occasion of his centenary.

The effect of the concentration of ammonia on the catalytic currents of bovine and human hemoglobin, cysteine, cystine and glutathione was studied, and the concentration of 3·5 mol l^{-1} was found optimal for analytical purposes. Mixtures of hemoglobin and cysteine exhibited catalytic wave positions suitable for their simultaneous quantitation. In a buffer containing 3·5M ammonia, the catalytic current of hemoglobin is virtually independent of the concentration of cysteine, and at a constant concentration of hemoglobin, the catalytic current of cysteine increases linearly with its concentration over the range studied. Statistical evaluation gave evidence that hemoglobin and cysteine can be determined simultaneously in the above buffer. When accounting for some interfering effects with respect to the catalytic currents of glutathione and cysteine, complexation phenomena and properties of the hemoglobin surface layer must be taken into consideration.

Catalytic waves discovered by Brdička[1] proved to be a sensitive analytical tool for individual proteins[2,3] as well as for a number of low-molecular-weight organic compounds containing thiol or disulfide groups[2,4,5]. The nonlinear dependence of catalytic currents on concentration for compounds containing SH or SS groups predestines it to the determination of low concentrations of such compounds. Data relating to the possibility of simultaneous determination of low-molecular-weight sulfur compounds and proteins containing the above groups, however, are lacking in the literature, perhaps because of the unfavourable position of the catalytic waves of these compounds in conventional ammoniacal buffers, i.e. 0·1M-NH_4Cl + 0·1M or 1·0M-NH_3 (ref.[2]).

In the Ni(II)–cysteine (Cys) system, the range of Cys concentrations where the catalytic wave can be used for analytical purposes was found wider if a higher concentration of ammonia was applied[6]. Also, in buffers containing ammonia in higher concentrations the relation between the number of thiol groups in molecules of different hemoglobins and the catalytic current was observed to be linear[7,8]. The aim of the present work was to check whether in such or similar conditions, hemo-

globins can be determined in the presence of sulfur-containing amino acids or peptides. A positive result would show promise for application of this approach also to other proteins in the presence of amino acids and peptides. This would enable one to investigate the process of hydrolysis or the action of different destructive factors causing degradation of proteins.

EXPERIMENTAL

Polarographic measurements were performed using an OH-105 polarograph (Radelkis, Budapest). Direct current (d.c.) polarograms were recorded with a two-electrode system. A Kalousek vessel, fitted with a large-surface (about 13·5 cm^2) saturated calomel electrode (SCE) as the anode, was used. The dropping mercury electrode (DME) was a bent tip capillary (46·2° with respect to the plumb-line) after Smoler[9]; its constants have been reported[7].

Alternating current (a.c.) polarograms were recorded with a three-electrode system. A platinum sheet (geometrical surface approximately 4 cm^2) served as the auxiliary electrode, an SCE was the reference electrode. The DME was a spindle capillary according to Novotný et al.[10], with a controlled drop time of 2·23 s. Its mercury flow rate was 1·483 mg s^{-1} and drop time in water 11·25 s at the working height of the mercury column 40 cm.

All measurements were performed at 298·16 ± 0·1 K (25°C); oxygen was removed from the solutions by purified nitrogen[11]. After recording the polarograms, the concentration of ammonia was controlled by volumetric determination[11].

Two hemoglobins (Hb) were used, viz. bovine (HbB) and human (HbH) hemoglobin. Supplied by Sigma (U.S.A.), they were twice crystallized and lyophilized. The preparation of the stock solutions and their standardization have been described[7,8]. The concentrations of hemoglobin are reported with respect to the tetramer, for which the mean molecular mass of 65 000 is adopted[12].

L-Cysteine (Cys), L-cystine (CySS) and oxidized glutathione (Glu) were obtained from Koch–Light (U.K.), Merck (F.R.G.) and Schuchardt (F.R.G.), respectively. Stock solutions of these compounds were prepared from precisely weighed samples, dissolved in deoxygenated 0·1M-HCl (Cys) or water (CySS, Glu) and stored under nitrogen in small, tightly closed bottles, accomodated in a refrigerator at 5°C. Samples were taken while passing nitrogen through the bottle. All the other chemicals were of reagent grade purity.

The catalytic waves of the hemoglobins were measured invariably from the residual current level, subtracting from it the limiting current of the cobalt wave, as read from a calibration curve for the given ammonia concentration. This approach was necessary because the limiting current of cobalt depends on the concentration of ammonia[13], and distortion of the cobalt wave by maxima and catalytic waves hinders its precise measurement.

For cysteine, cystine and glutathione, only the sum wave, measured from the level of the limiting current of the cobalt wave, was evaluated unless stated otherwise.

The concentrations of NH_4Cl and $CoCl_2$ were 0·1 mol l^{-1} and 5·2 . 10^{-4} mol l^{-1}, respectively, in all solutions used for studying the catalytic waves.

RESULTS

The measurements were performed using bovine and human hemoglobins, which contain different numbers of SH groups in the tetramers (2 in HbB and 6 in HbH).

Cysteine, cystine and glutathione served as low-molecular weight compounds containing SH or SS groups.

The results of qualitative investigation of the effect of concentration of ammonia on the position of the catalytic waves of the compounds studied are presented in Fig. 1. Data for cystine are not included because they are similar to those of cysteine. A comparison of the wave positions indicates that the first hemoglobin wave cannot be used for quantitation in buffers containing ammonia in concentrations as low as 0·1 or 1·0 mol l^{-1}. In these conditions, the catalytic waves of hemoglobin and cysteine or glutathione overlap. The optimum concentration of ammonia is 3·5 mol . l^{-1}, the first hemoglobin wave being then disturbed by the catalytic waves of Cys and Glu to the least extent. At the same time, the maximum of Cys and Glu waves appears at a potential, where a minimum occurs after the catalytic wave of Hb. This shows promise for the quantitation of Cys, CySS and Glu as well.

The effect of ammonia on the catalytic currents was also studied (Figs 2, 3). The catalytic current of hemoglobins (Fig. 2) is affected in a manner which is quite different from that in which the catalytic currents of Cys, CySS and Glu are affected (Fig. 3). The very low catalytic current of glutathione is particularly noteworthy (Fig. 3, curve *3*); actually, its concentration used, $2 . 10^{-4}$ mol l^{-1}, was about 130 times higher than that of the remaining compounds ($1{\cdot}54 . 10^{-6}$ mol l^{-1}).

With regard to the favourable result of the qualitative investigation, the buffer containing ammonia in a concentration of 3·5 mol l^{-1} was applied in the additional study of the two hemoglobins and the remaining compounds.

The dependences of the catalytic currents on the concentration of the compounds examined are shown in Figs 4 and 5. In the case of the hemoglobins the curve shapes are typical of proteins (Fig. 4). For Cys, CySS and Glu, on the other hand, the catalytic currents increase strictly linearly with their concentration over the range studied; the correlation coefficients of the regression straight lines attain values as high as 0·9994, 0·9987 and 0·9987, respectively.

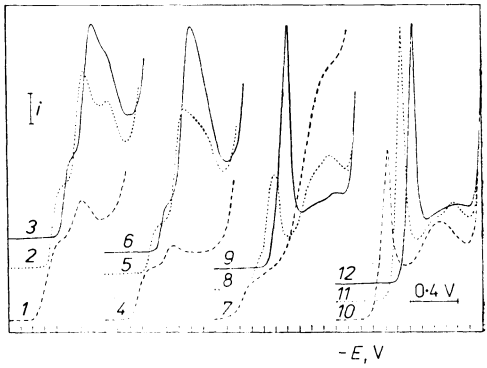

FIG. 1

Effect of concentration of ammonia on the shape of catalytic waves of bovine hemoglobin (*1*—*3*), human hemoglobin (*4*—*6*), glutathione (*7*—*9*) and cysteine (*10*—*12*). Concentrations (mol l^{-1}): Hb $1{\cdot}54 . 10^{-6}$, Cys $1{\cdot}54 . 10^{-6}$, Glu $2 . 10^{-4}$, NH_3 0·1 (*1*, *4*, *7*, *10*), 1·0 (*2*, *5*, *8*, *11*), 3·5 (*3*, *6*, *9*, *12*). All polarograms start at −0·8 V vs SCE. Calibration current *i* corresponds to 1 μA (HbB), 1·6 μA (HbH, Cys), 1·8 μA (Glu)

Mixtures of hemoglobin and cysteine were studied at concentrations of $0{\cdot}5 . 10^{-6}$ mol l^{-1} (HbB or HbH) or $1{\cdot}54 . 10^{-6}$ mol l^{-1} (HbB or HbH), and $0-1 . 10^{-5}$ mol l^{-1} (Cys). The effect of cysteine on the shape of catalytic waves of hemoglobin is qualitatively shown in Fig. 6, demonstrating that the first catalytic wave of hemoglobins (wave I, Fig. 6*a*–*d*) is well measurable in all circumstances. Only at the lowest concentration of HbB and the highest concentrations of Cys (Fig. 6*a*, curves *4*, *5*) the limiting current of wave I is somewhat poorer in shape. Wave II, which is the sum of the second hemoglobin wave and the first cysteine wave, was not employed because of difficulties associated with its measurement. On the other hand, wave III, which is the sum wave of cysteine, is easy to measure in all circumstances (from the minimum current level of the catalytic waves of hemoglobins, at about $-1{\cdot}8$ V vs SCE).

Quantitative results concerning the effect of cysteine on hemoglobin wave I are presented in Table I. The dependence of wave III on the concentration of cysteine is shown in Fig. 7. Wave III depends linearly on the concentration of Cys over the entire concentration range studied. This is documented by high values of the regres-

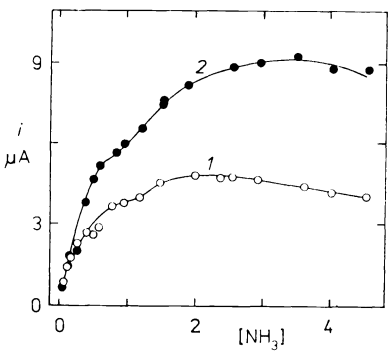

FIG. 2

Dependence of the catalytic current of bovine (*1*) and human (*2*) hemoglobin on the concentration of ammonia (mol l^{-1}). Hemoglobin concentration $1{\cdot}54 . 10^{-6}$ mol l^{-1}

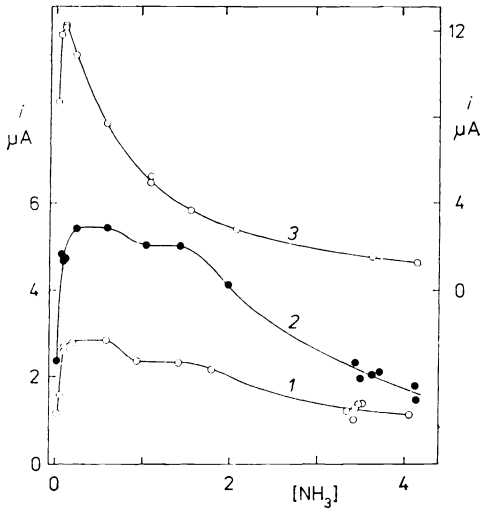

FIG. 3

Dependence of the catalytic current of cysteine (*1*), cystine (*2*) and glutathione (*3*) on the concentration of ammonia (mol l^{-1}). Concentrations (mol l^{-1}): Cys and CySS $1{\cdot}54 . 10^{-6}$, Glu $2 . 10^{-4}$. The left-hand scale applies to Cys and CySS, the right-hand scale, to Glu

sion correlation coefficients, which are 0·9979, 0·9997, 0·9963 and 0·9978, respectively. The slope of these lines, however, depends on the kind of the hemoglobin as well an on its concentration (Table II).

DISCUSSION

Results of the preliminary examination (Fig. 1) showed that the assumptions that stimulated the present work were correct. Increase in the ammonia concentration brings about a shift of the catalytic waves of all the compounds studied to more negative potentials, although to different degrees. This is due to the catalyzed evolution of hydrogen as well as to the formation of complexes of the compounds studied with cobalt ions, which are active in this process. The fact that the conditions are favourable for the simultaneous determination of hemoglobin and cysteine is a result obtained into the bargain.

The sensitivity of the determination of hemoglobin is only satisfactory at high concentrations of ammonia (Fig. 2). This is associated with the increased accessibility of the thiol groups in the hemoglobins resulting from their decomposition into dimers[15]. The catalytic currents of cysteine, cystine and glutathione, however, decrease

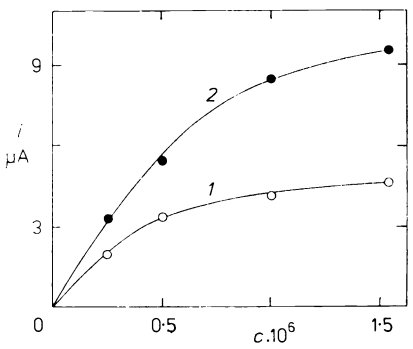

FIG. 4

Dependence of the catalytic currents of bovine (*1*) and human (*2*) hemoglobin on their concentration (mol l^{-1}) in a buffer containing ammonia in a concentration of 3·5 mol l^{-1}

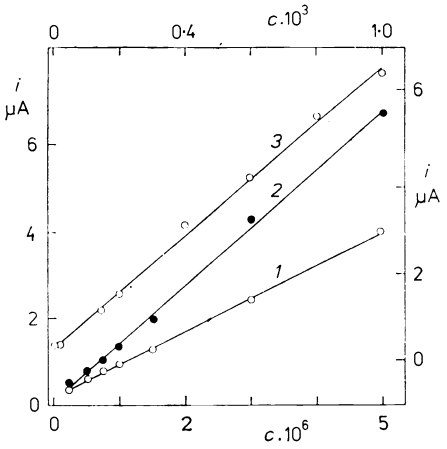

FIG. 5

Dependence of the catalytic currents of cysteine (*1*), cystine (*2*) and glutathione (*3*) on their concentration (mol l^{-1}) in a buffer containing ammonia in a concentration of 3·5 mol l^{-1}. The left-hand and lower scales apply to Cys and CySS, the right-hand and upper scales, to Glu

considerably in such conditions due to the action of ammonia as a competing ligand (Fig. 3). Nevertheless, the strictly linear dependence of the catalytic current on the concentration of Cys. CySS and Glu over the range studied can be useful for analytical applications (Fig. 5). Figure 3 shows the ammonia concentration range over which the catalytic current ratio for isomolar solutions of cystine and cysteine is 2 : 1 (ref.[2]).

On the other hand, some limitations were also revealed. The optimum range of concentrations of hemoglobins for their determination follows from the shape of the curves shown in Fig. 4. Additional measurements with cystine were not accomplished because of the instability of its solutions[16,17]. Glutathione was rejected as well since at concentrations comparable with those of the other compounds studied, its catalytic current is undetectable (Fig. 3). The high effect of ammonia on the catalytic currents of glutathione as compared to cysteine (Fig. 3) is apparently associated with complexation of Co(II). Complexes of cysteine are very stable[18], and the nearly identical catalytic currents of cystine, which is reduced[19] before the appearance

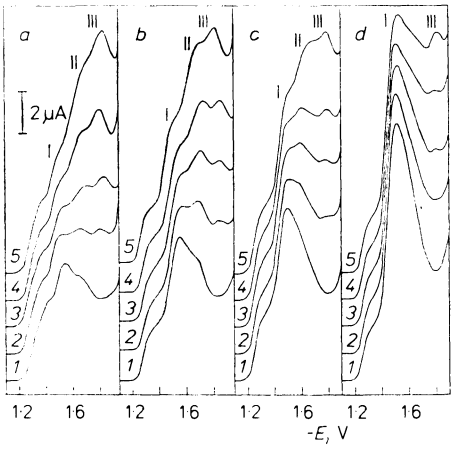

FIG. 6

Effect of the concentration of cysteine on the shape of catalytic waves of bovine (*a*, *b*) or human (*c*, *d*) hemoglobin. Cysteine concentration (μmol l^{-1}): *1* 0, *2* 3, *3* 5, *4* 7, *5* 10. Concentration of ammonia in the buffer 3·5 mol l^{-1}

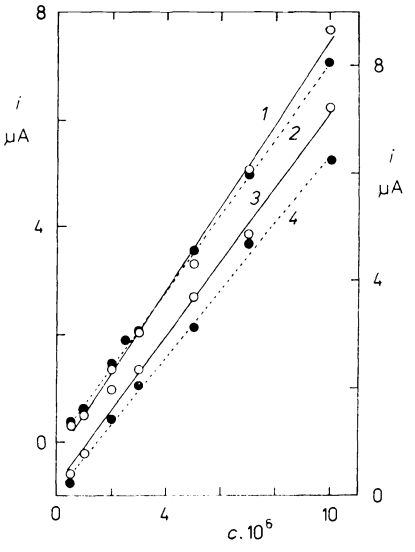

FIG. 7

Dependence of the catalytic current of cysteine on its concentration (mol l^{-1}) in the presence of bovine (*1*, *2*) and human (*3*, *4*) hemoglobin. Concentrations (mol l^{-1}): hemoglobin $0{\cdot}5 \cdot 10^{-6}$ (*1*, *3*) or $1{\cdot}54 \cdot 10^{-6}$ (*2*, *4*), ammonia in the buffer 3·5. The left-hand scale applies to bovine hemoglobin, the right-hand scale, to human hemoglobin

TABLE I

The effect of cysteine on the catalytic currents of bovine and human hemoglobin in a buffer containing 3·5M-NH_3[a]

Δ[Cys][b] μmol l^{-1}	n^c	i^d μA	s^e μA	s^e %	Rel. int.[f], μA (1 − α)[g] 0·95	Rel. int.[f], μA (1 − α)[g] 0·99
			0·5 . 10^{-6}M HbB			
0—5	6	2·821	0·097	3·4	0·097	0·153
0—10	8	2·793	0·106	3·8	0·088	0·131
			1·54 . 10^{-6}M HbB			
0—5	7	4·151	0·068	1·6	0·060	0·093
0—10	9	4·089	0·171	4·2	0·131	0·186
			0·5 . 10^{-6}M HbH			
0—5	6	5·589	0·056	1·0	0·056	0·089
0—10	8	5·570	0·073	1·3	0·060	0·089
			1·54 . 10^{-6}M HbH			
0—5	6	9·585	0·137	1·4	0·137	0·216
0—10	8	9·576	0·120	1·3	0·099	0·147

[a] Standard deviations and reliability intervals were calculated using the method of range[14]; [b] range of cysteine concentration; [c] number of experimental points; [d] mean value of i; [e] standard deviation, [f] reliability interval, [g] significance level.

TABLE II

Values of the slope of the regression line $i = a + b$ [Cys] in a buffer containing 3·5M-NH_3 and different concentrations of bovine or human hemoglobin

[Hb] μmol l^{-1}	In the presence of HbB			In the presence of HbH		
	b^a	s^b	r^c	b^a	s^b	r^c
0	0·757	0·012	0·9994	0·757	0·012	0·9994
0·5	0·776	0·023	0·9979	0·692	0·027	0·9963
1·54	0·711	0·007	0·9997	0·629	0·021	0·9978

[a] slope, μA/μmol l^{-1}; [b] standard deviation of the slope; [c] correlation coefficient.

of the catalytic wave, give evidence of a rapid formation of the Co(II)–Cys complex Oxidized glutathione is also reduced[2] before the catalytic wave. The Co(II) complexes formed by the oxidized form of glutathione involve coordination sites of the donor different from those occurring if glutathione is present in its reduced form[20]. It cannot be decided whether the low catalytic activity of Glu is due to steric or kinetic factors in the formation of the active complex or whether its formation constant is considerably lower, as in the case of complexes of Co(II) with the Ala-Cys dipeptide[21].

Data in Table I document that the proposed composition of the ammonia buffer is very suitable for the determination of hemoglobins in the presence of cysteine. The standard deviation values and the reliability intervals testify to a high precision of the determination, taking into account the very low concentration involved. Table I also demonstrates that over the range studied, cysteine virtually does not affect the mean catalytic current of hemoglobin (compare the third column in Table I); the differences lie within the stanard deviation limits.

The results presented in Fig. 7 and Table II demonstrate that cysteine can be determined in the presence of hemoglobin. The slope of the regression line, however, depends on the concentration and kind of hemoglobin (Table II). This indicates that the surface layer of hemoglobin affects the catalytic process in which cysteine is involved. This conclusion is borne out by the a.c. polarographic data (Fig. 8). To ensure similar experimental conditions, the controlled drop time was chosen close to that applied in the studies of catalytic currents. The difference between the DME capacity in the buffer in the absence (C^0) and in the presence of hemoglobin (C), $\Delta C = C^0 - C$, increases with increasing hemoglobin concentration. In the presence of either of the hemoglobins in a concentration of $1{\cdot}5 \cdot 10^{-6}$ mol l^{-1}, the formation of the surface layer is nearly complete (Fig. 8). The slopes of the cysteine catalytic current regression lines depend on the kind of hemoglobin present. This suggests that the surface layers of bovine and human hemoglobins have different structures.

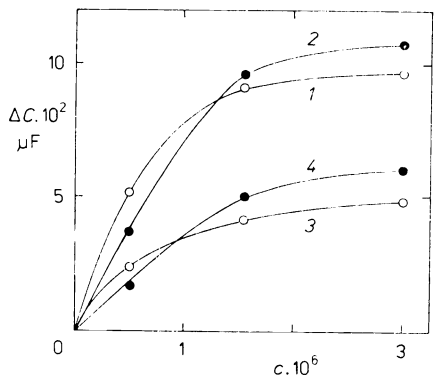

FIG. 8

Dependence of the capacity difference ΔC (see text) of the DME on the concentration of bovine (*1*, *3*) and human (*2*, *4*) hemoglobin in a buffer containing ammonia in a concentration of 3·5 mol l^{-1} in the absence of cobalt ions. Potential (V): *1* −0·6, *2* −0·6, *3* −0·8, *4* −0·8 (vs SCE)

The fact, that in spite of a considerable adsorption of hemoglobin, the catalytic process involving cysteine is only slightly affected (compare lines *2* and *1*, and *4* and *3*) suggests that the surface layer is not very compact. Otherwise a charge transfer across the adsorption layer, similar to that proposed for some proteins[22,23], should be considered. The relatively not very marked effect of Hb on the catalytic currents of cysteine cannot be associated with the adsorption of the latter or of its complexes with Co(II). At higher ammonia concentrations the surface activity of cysteine (or its complexes with Co(II)) vanishes altogether. This is apparent from the abrupt increase of the first kind maximum on the cobalt wave (Fig. 1, curves *10*–*12*), similar to the case of glutathione (Fig. 1, curves *7*–*9*) and cystine.

In summary, the results obtained give evidence that the proposed composition of the buffer solution is suitable for simultaneous determination of hemoglobin and cysteine. Some additional, unpublished data suggest that this may apply to the simultaneous determination of other proteins and cysteine (or cystine) as well.

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