# STUDY OF PROTEIN REACTIVITY WITH THIOL REAGENTS BY ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The binding of a thiol reagent to a protein modifies its chromatographic behaviour when the reagent changes the charge of the protein. Consequently, the kinetics of mixed disulphide formation can be determined by high-performance liquid chromatography, which allows the measurement of the amounts of unreacted and reacted protein fractions. The conditions of chromatographic separation have been studied on Synchropak AX-300 columns. The separation depends essentially on the charge of the compound added to the protein, pH and ionic strength. The reactivity of several disulphides with human haemoglobin and with human and bovine serum albumin have been determined. For uncharged disulphides a method based on the competition of a charged disulphide of a known reactivity in the presence of an uncharged one was used. This technique appears to be a general, simple and fast means of comparing the reactivity of a protein thiol towards various reagents.

INTRODUCTION

The problem of covalent modification of a protein is of biological importance in the case of haemoglobin (Hb). We have previously shown[1] that the modification of the thiol function of Cys $\beta$ 93 by different specific reagents affords an important change in the functional properties of haemoglobin. This point has been investigated for the evaluation of antisickling drugs. The reversible drug or ligand binding of proteins has been studied by different high-performance liquid chromatographic (HPLC) methods, which have been reviewed[2,3]. The covalent fixation of a reactive compound on a protein has been studied much less. One example is the work of Strömme[4], who studied the reaction of tetraethylthiuram disulphide with serum proteins. The chromatography of the reaction mixture on Sephadex G-25 gives three peaks, the first being related to reacted and unreacted protein and the other two to disulphide and thiol.

The speed of HPLC analysis suggests the use of this technique in order to

determine the kinetic parameters of the fixation. This work was devoted to the study of the reaction of different disulphides with some proteins (including haemoglobin).

The use of Synchropak AX-300 resin gives a rapid separation[5-14] of reacted from unreacted protein, permitting the evaluation of the extent of reaction after short reaction times. This process differs from previous ones based on a spectroscopic method, which measure the thiol release in only a few instances.

## EXPERIMENTAL

### HPLC

Two 6000 A pumps from Water Assoc. (Milford, MA, U.S.A.) were monitored with a Waters Assoc. Model 660 solvent programmer gradient and were connected to a Rheodyne (Berkeley, CA, U.S.A.) 7125 injector. The injection volume (loop) was 0.05 ml. The column (5 cm × 7 mm I.D.) was filled in our laboratory with Synchropak (Synchrom, Linden, IN, U.S.A.) AX-300 anion exchanger (pore diameter 300 Å, bead diameter 10 $\mu$m). A Pye Unicam (Cambridge, U.K.) Type LC3 detector was used for the absorbance measurements at 415 nm for Hb proteins and 280 nm for the albumin proteins.

### Proteins

Haemolysate was prepared from freshly drawn red blood cells, collected in sodium chloride, EDTA and glucose media. The red blood cells were washed three times with isotonic saline solution, lysed in water and centrifuged in order to remove cell membranes. Then, haemoglobin solution was stripped of small thiol compounds by dialysis, repeated five times, against 50 volumes of buffer at 4°C, and was passed through a Sephadex G-25 column to remove all derivatives that could interfere with the haemoglobin thiol groups. The glutathione derivative of Hb and Hb–S-ethylamine were obtained as described previously[1,15].

Human and bovine serum albumin were obtained from Sigma (St. Louis, MO, U.S.A.). Before use, these albumins were also stripped by dialysis.
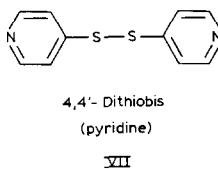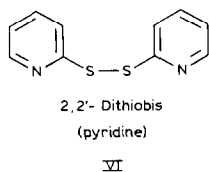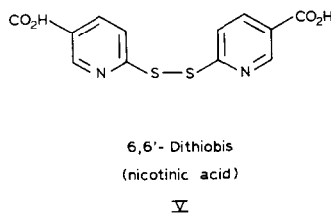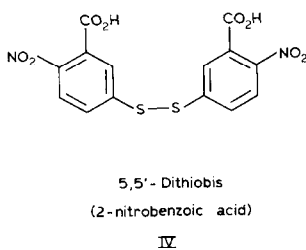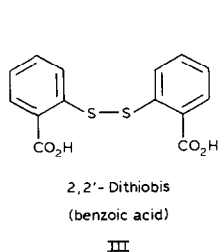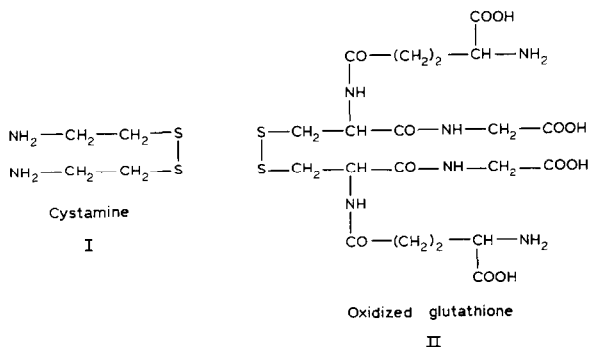
### Materials

Cystamine dichloride was obtained from Merck (Darmstadt, F.R.G.). Oxidized glutathione, 2,2'-dithiobis(benzoic acid), and 5,5'-dithiobis(2-nitrobenzoic acid) were purchased from Janssen (Pantin, France). 2,2'-Dithiobis(pyridine), 4,4'-dithiobis(pyridine) and 6,6'-dithiobis(nicotinic acid) were purchased from Aldrich (Strasbourg, France). Other chemicals were of analytical-reagent grade.

### Procedures

The binding kinetics of disulphide compounds on protein were studied in 20 m$M$ Tris buffer (pH 7.4) at 25°C in a water-bath shaker. For each reaction time (the shortest was 30 s) a reaction vessel was filled. A sample of 50 $\mu$l of each mixture was injected into the chromatographic system. The time needed for sample transfer and injection was less than 10 s. The chromatographic column was maintained at 25°C with a thermostated jacket. The flow-rate was 2 ml/min and the eluent composition was as described below.

*First method.* The reaction of disulphides bearing a charged moiety with hae-

Cystamine

I

Oxidized glutathione

II

2,2'-Dithiobis (benzoic acid)

III

5,5'-Dithiobis (2-nitrobenzoic acid)

IV

6,6'-Dithiobis (nicotinic acid)

V

2,2'-Dithiobis (pyridine)

VI

4,4'-Dithiobis (pyridine)

VII

moglobin induces a modification of protein charge allowing, in many instances, a chromatographic separation from unmodified Hb on a Synchropak AX-300 column. The concentration of each kind of protein can be easily obtained from peak areas.

From the determination of these proteins we are able to measure the rate of reagent fixation according the following equation:

$$\text{P–SH} + \text{M–S–S–M} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons} } \text{P–S–S–M} + \text{M–SH}$$
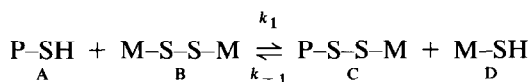
where P–SH (A) is the protein of interest, M–S–S–M (B) is the charged disulphide, P–S–S–M (C) is the modified protein and M–SH (D) is the thiol or thione generated.

The rate of P–S–S–M (C) formation is

$$\frac{d\,[\text{C}]}{dt} = k_1[\text{A}]_t[\text{B}]_t - k_{-1}[\text{C}]_t[\text{D}]_t \tag{1}$$

where $[A]_t$, $[B]_t$, $[C]_t$ and $[D]_t$, are the concentrations at time $t$. Let

$$[A]_t = [A]_0 - x = [A]_e - y$$
$$[B]_t = [B]_0 - x = [B]_e - y$$
$$[C]_t = [C]_0 + x = [C]_e + y$$
$$[D]_t = [D]_0 + x = [D]_e + y$$

where $[A]_0$, $[B]_0$, $[C]_0$ and $[D]_0$ are the concentrations at zero time and $[A]_e$, $[B]_e$, $[C]_e$ and $[D]_e$ are the concentrations at equilibrium. Eqn. 1 gives

$$\frac{dy}{dt} = k_1 \{([A]_e - y)([B]_e - y)\} - k_{-1} \{([C]_e + y)([D]_e + y)\}$$

as at equilibrium

$$K_{eq} = \frac{[C]_e[D]_e}{[A]_e[B]_e} = \frac{k_1}{k_{-1}}$$

$$\frac{dy}{dt} = y^2 (k_1 - k_{-1}) - y \{k_1 ([A]_e + [B]_e) + k_{-1} ([C]_e + [D]_e)\} \qquad (2)$$

if

$$a = k_1 - k_{-1}$$

and

$$b = - \{k_1 ([A]_e + [B]_e) + k_{-1} ([C]_e + [D]_e)\}$$

then eqn. 2 can be written as

$$\frac{dy}{dt} = ay^2 + by$$

After integration, we obtain

$$\log \left( a + \frac{b}{y} \right) = -bt + Constant$$

As at time $t = 0$, $y = [A]_e - [A]_0$, this becomes

$$\log \left( a + \frac{b}{y} \right) = -bt + \log \left( a - \frac{b}{c} \right) \qquad (3)$$

where $c = [A]_0 - [A]_e = x - y$

Let

$$d = \frac{b}{k_1} = -\left\{[A]_e + [B]_e + \frac{k_{-1}}{k_1}([C]_e + [D]_e)\right\}$$

and

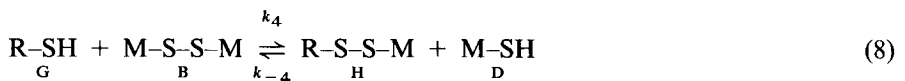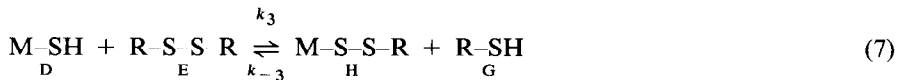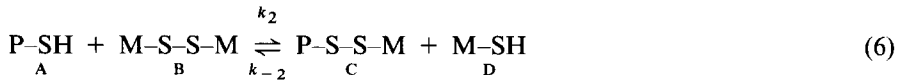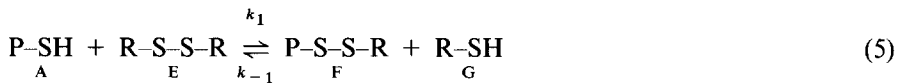$$e = \frac{a}{k_1} = 1 - \frac{k_{-1}}{k_1}$$

Eqn. 3 then gives

$$\log\left(e + \frac{d}{x - c}\right) = -k_1 dt + \log\left(e - \frac{d}{c}\right) \tag{4}$$

By plotting $\log\left(e + \dfrac{d}{x - c}\right)$ as a function of time, $t$, we obtain a straight line (*cf.*, eqn. 4) with slope $-k_1 d$, allowing the determination of $k_1$ after calculation of $c$, $d$ and $e$ from the measurement of $[A]_e$, $[B]_e$, $[C]_e$ and $[D]_e$.
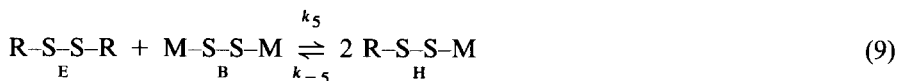
This method has been used in each instance where the complete chromatographic separation of modified and unmodified proteins was possible.

*Second method.* When the protein reacted with a disulphide could not be separated from the initial protein, we used another method based on the simultaneous reaction of two disulphides with the protein. One of these disulphides (M–S–S–M), considered as a marker, actually gives a protein bearing a mixed-charged disulphide separable from the initial protein by the AX-300 column.

The rate of the marker reaction is affected by the reactivity of the other disulphide in the same protein. Consider all the possible reactions:

$$\underset{A}{P-SH} + \underset{E}{R-S-S-R} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{F}{P-S-S-R} + \underset{G}{R-SH} \tag{5}$$

$$\underset{A}{P-SH} + \underset{B}{M-S-S-M} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{C}{P-S-S-M} + \underset{D}{M-SH} \tag{6}$$

$$\underset{D}{M-SH} + \underset{E}{R-S-S-R} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \underset{H}{M-S-S-R} + \underset{G}{R-SH} \tag{7}$$

$$\underset{G}{R-SH} + \underset{B}{M-S-S-M} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \underset{H}{R-S-S-M} + \underset{D}{M-SH} \tag{8}$$

Reaction $9 = 7 + 8$, and hence

$$R\text{–}S\text{–}S\text{–}R \ + \ M\text{–}S\text{–}S\text{–}M \ \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \ 2\ R\text{–}S\text{–}S\text{–}M \tag{9}$$
$$\underset{E}{} \qquad\qquad \underset{B}{} \qquad\qquad\qquad \underset{H}{}$$

Reactions of mixed disulphides containing proteins P–S–S–M and P–S–S–R with thiol are the sums of reactions $6 + 8$ and $5 + 7$.

In the initial period of the reaction, the concentrations of D and G are very low, as B and E are in excess with respect to the protein. Consequently, reactions 7 and 8 can be neglected. Moreover, for short reaction times, the effect of reverse reactions in eqns. 5 and 6 are also negligible. This hypothesis has been experimentally verified (see Results).

Under these conditions

$$-\frac{d[A]}{dt} = \frac{d[F]}{dt} + \frac{d[C]}{dt} = k_1[A]_t[E]_t + k_2[A]_t[B]_t$$

Owing to the excess of B and E, and for short reaction times, it can be assumed that $[B]_t = [B]_0$ and $[E]_t = [E]_0$, and then

$$-\frac{d[A]}{[A]_t} = (k_1[E]_0 + k_2[B]_0)dt$$

After integration,

$$[A]_t = [A]_0 \exp\{-(k_1[E]_0 + k_2[B]_0)t\} \tag{10}$$

As we are able to follow by HPLC the formation of C, the concentration of this compound can be calculated from

$$\frac{d[C]}{dt} = k_2[A]_t[B]_0$$

From eqn. 10

$$\frac{d[C]}{dt} = k_2[A]_0[B]_0 \exp\{-(k_1[E]_0 + k_2[B]_0)t\}$$

By integration, we obtain

$$[C]_t = \frac{k_2[A]_0[B]_0\ (1 - \exp\{-(k_1[E]_0 + k_2[B]_0)t\})}{k_1[E]_0 + k_2[B]_0} \tag{11}$$

As $k_2$ is known from the first method, $k_1$ can be calculated for different values of measured $[C]_t$ at different short times. Depending on the different compounds, we made three or five determinations to obtain $k_1$. The variation was within an error of 5%.

*Electrophoresis*

The extent of modification of haemoglobin by charged reagents could be also assessed by isoelectric focusing on slabs of acrylamide gels, performed as described previously[1].

Modified haemoglobins appeared as focused bands, distinct from that of un-modified haemoglobin. The percentage of modification was determined by densito-metry with a Cellosystem (Sebia, Issy les Moulineaux, France), as previously de-scribed[16]. The results were evaluated as in the first chromatographic method.

RESULTS

*Separation conditions*

We first studied the isocratic elution of Hb $A_0$ from the Synchropak AX-300 column at different pH values. From Fig. 1, it appears that Hb $A_0$ is retained above pH 7.8. For the kinetic determinations, it is necessary that the reaction of Hb and disulphides be stopped when the sample is injected for the chromatographic sepa-ration. We chose pH 8.0 for the subsequent experiments, as this value allows a slight but sufficient retention of the Hb $A_0$ and the separation of reagents. Under these conditions, we observed that uncharged and positively charged low-molecular-weight compounds were excluded from the column and appeared at the void volume, while negatively charged disulphides were more strongly retained than Hb.

We measured the effect of different salt gradients as the eluent on the sepa-ration of Hb $A_0$ and Hb–glutathione, considered to be a model of modified Hb $A_0$ with a negatively charged adduct. The experiments were performed with different sodium salts: sulphate, chloride, formate and acetate. The ionic strength ($\mu = 0.1$) was the same for the different concentrated salt solutions used to obtain the gradients.
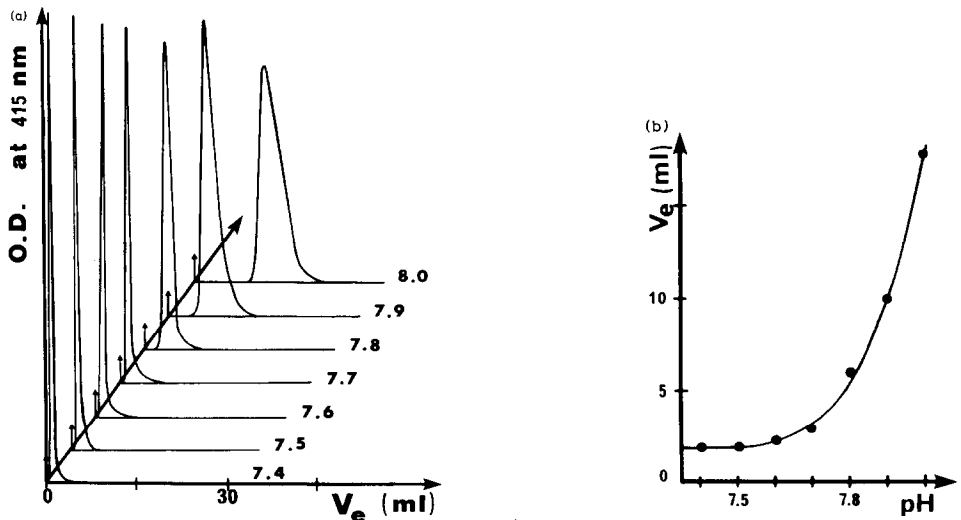


Fig. 1. (a) Effect of eluent pH on the isocratic elution of Hb $A_0$, detected at 415 nm. Eluent, 0.02 $M$ Tris buffer (pH 7.4–8.0); flow-rate, 2 ml/min; injection volume, 50 $\mu$l; temperature, 25°C. (b) Variation of the elution volume of the Hb $A_0$ peak as a function of the eluent pH.
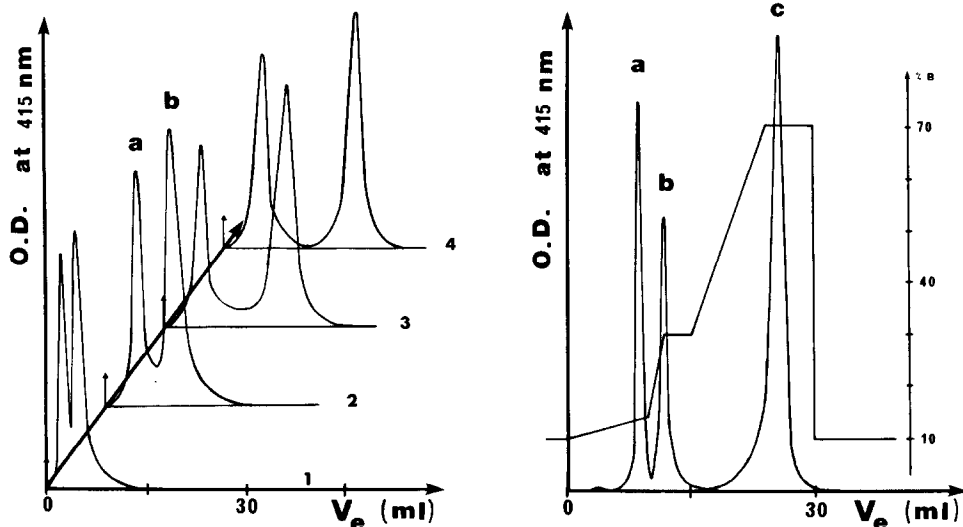
Fig. 2. Effect of salt gradient composition on the separation of (a) Hb $A_0$ and (b) Hb–glutathione, detected at 415 nm. Eluent, linear gradient from 10% to 90% B in 15 min; A, 0.02 $M$ Tris buffer (pH 8.0); B, 0.02 $M$ Tris buffer (pH 8.0) with different sodium salts ($\mu = 0.1$): 1 = sulphate, 2 = chloride, 3 = formate, 4 = acetate; flow-rate, 2 ml/min; injection volume, 50 $\mu$l; temperature, 25°C.

Fig. 3. Separation of Hb (b) from haemoglobin, modified with a positively charged group (a), or a negatively charged group (c). (a) Hb–S-ethylamine; (b) Hb $A_0$; (c) Hb–glutathione. Eluent, gradient from 10% to 70% B in 15 min; A, 0.02 $M$ Tris buffer (pH 8.0); B, 0.02 $M$ Tris buffer (pH 8.2) with 0.1 $M$ sodium acetate; flow-rate, 2 ml/min; injection volume, 50 $\mu$l; temperature, 25°C.

The results are presented in Fig. 2. It appears that acetate ions allow the best separation.

In order to separate modified Hb $A_0$, tested simultaneously with two disulphides (I and II), we determined the optimal conditions by changing the gradient shape. The experiments in Fig. 3 were performed with Hb $A_0$, Hb–glutathione and Hb–S-ethylamine.

The results showed the dependence of the charge of the protein when Cys 93 residue of Hb $A_0$ is modified. A cationic adduct lowers the retention (Hb–S-ethylamine), whereas the retention is increased by an anionic moiety (Hb–glutathione).

The influence of pH on the elution of human serum albumin (HSA) from Synchropak AX-300 is shown in Fig. 4. Under the experimental conditions used, retention began above pH 4.6.

For the separation of modified HSA with a negatively charged adduct (II) from unmodified HSA, we have chose pH 6.4 (phosphate buffer) with acetate as the eluent ion, as with haemoglobin (Fig. 5). The same conditions were selected for the study of bovine serum albumin (BSA).

*Determination of kinetic parameters of the protein disulphide reaction*

We used the first method described above for disulphides bearing a positively or a negatively charged group, which is transferred to the protein and changes the chromatographic behaviour. We studied the following compounds: cystamine (I),
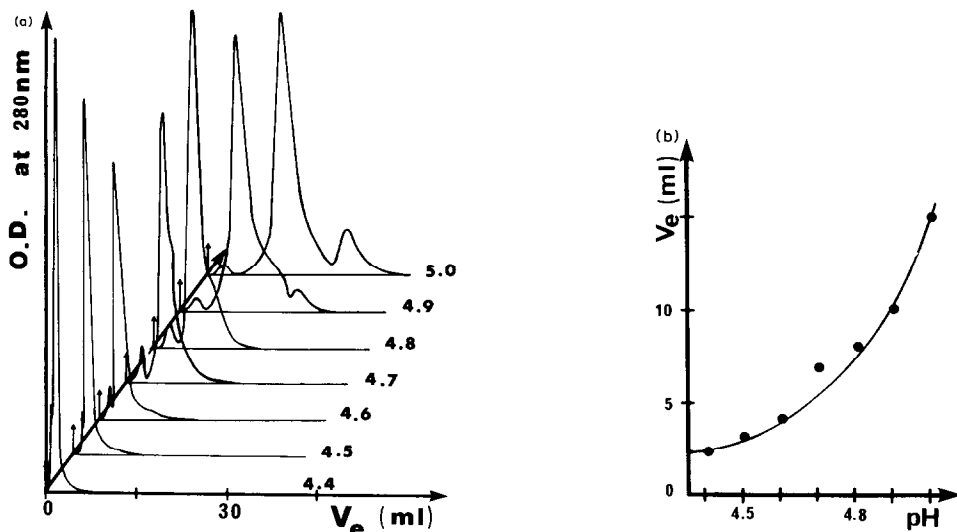
Fig. 4. (a) Effect of eluent pH on the isocratic elution of HSA, detected at 280 nm. Eluent, 0.02 $M$ acetate buffer (pH 4.4–5.0); flow-rate, 2 ml/min; injection volume, 50 $\mu$l; temperature, 25°C. (b) Variation of the elution volume of the HSA peak as a function of the eluent pH.

oxidized glutathione (II), 2,2'-dithiobis(benzoic acid) (III), 5,5'-dithiobis(2-nitroben-zoic acid) (IV) and 6,6'-dithiobis(nicotinic acid) (V).

The spectrophotometric titration of the free sulphydryl groups of haemoglobin was obtained as described previously[17–20] by reacting Hb $A_0$ with IV or V in larger excess ($r$ = RSSR/Hb = 40).

The modified protein, measured after a reaction time of 2 h at 25°C, gave a value of 1.96 SH per mole, very close to the theoretical value. Identical measurements gave 0.49 for HSA and 0.58 for BSA, close to the values of Pedersen and Jacobsen[21] (HSA 0.45, BSA 0.58). The production of only *ca.* 0.5–0.6 equivalents of SH per mole of albumin has been observed previously[22–24] and is due to 40% of the protein existing as a mixed disulphide of cysteine and glutathione[22].

The possible reaction of these mixed disulphide-containing proteins with thiol or thione released from reaction with disulphide reagent has not been considered. This reaction could occur after a long reaction time as in the first method. From experiments obtained after very different reaction times (2 min–2 h) it appears that the calculated rate constants are good agreement. Moreover, we verified by spec-troscopy that 2-pyridinethione does not react with crude HSA or BSA. This property can be extended to other highly reactive disulphides. For these reasons, the presence of an initial mixed disulphide of HSA or BSA has been neglected in the calculations.

*Reaction order*

For short reaction times, the reverse reaction can be neglected, and the rate equation is

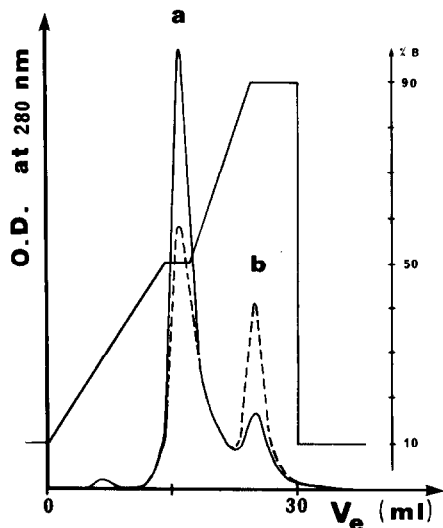$$\frac{d[C]}{dt} = k_1[A]^a[B]^b = v$$

Fig. 5. Separation of HSA (a) from HSA modified with a negatively charged group (b), detected at 280 nm. Eluent, gradient from 10% to 90% B in 15 min; A, 0.02 $M$ phosphate buffer (pH 6.4); B, 0.02 $M$ phosphate buffer (pH 6.4) with 0.3 $M$ sodium acetate; flow-rate, 2 ml/min; injection volume, 50 $\mu$l; temperature, 25°C.
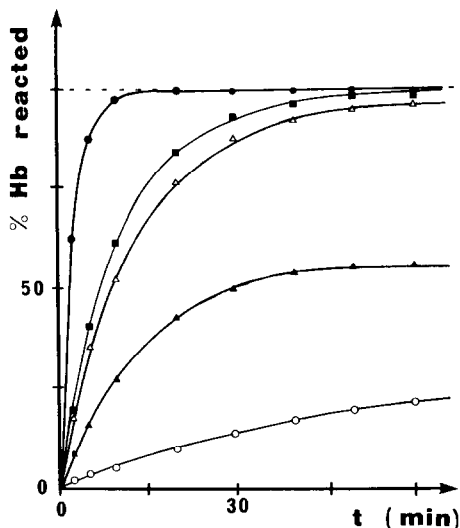
Fig. 6. Time course of the reaction of haemoglobin in solution with charged disulphides. I, Cystamine (△); II, oxidized glutathione (○); III, 2,2'-dithiobis(benzoic acid) (▲); IV, 5,5'-dithiobis(2-nitrobenzoic acid) (●); V, 6,6'-dithiobis(nicotinic acid) (■). Incubations were performed at 25°C in 0.02 $M$ Tris buffer (pH 7.4). [Hb $A_0$] = $10^{-5}$ $M$; [R–S–S–R] = $2 \cdot 10^{-4}$ $M$.

If $-\log v$ is plotted as a function of $-\log [B]$, we can obtain $b$ from the slope; for V, $b = 1.09 \pm 0.05$. We thus verified that the binding reaction of disulphide on Hb is first order. We ascertained the same for the other compounds and proteins.

*Kinetic parameters*

Fig. 6 shows the extent of reaction as a function of time for different charged disulphides. We were able to obtain significant measurements for reaction times as short as 2 min. Generally the measurements were obtained after 5 min in order to avoid errors due to the mixing time.

For the same disulphide/protein ratio we obtained large differences in the reaction kinetics for various disulphides, as shown in Fig. 6. By application of the first method described above, we found the rate constants listed in Table I.

In order to verify the validity of the second method, the first direct and the competition methods were compared by using two charged disulphides, I and V, with Hb $A_0$. The value of $k_1$ for I obtained by application of eqn. 11 (940 $\pm$ 50 min$^{-1}$) was in good agreement with the value obtained by the first method (990 $\pm$ 50 min$^{-1}$).

The results for $k_1$ measured for the uncharged disulphides VI and VII by the competition method are listed in Table II. These results can be compared with those previously obtained from electrophoresis measurements. The comparison of the ki-

TABLE I

KINETIC CONSTANTS DETERMINED BY THE FIRST DIRECT METHOD (cf. EQN. 4) FOR FIVE DISULPHIDES AND HAEMOGLOBIN, HUMAN SERUM ALBUMIN AND BOVINE ALBUMIN

Disulphides used: I, cystamine; II, oxidized glutathione; III, 2,2'-dithiobis(benzoic acid); IV, 5,5'-dithiobis(2-nitrobenzoic acid); V, 6,6'-dithiobis(nicotinic acid). Proteins used: Hb, haemoglobin; HSA, human serum albumin; BSA, bovine serum albumin. The binding kinetic of disulphide on protein was performed in 0.02 $M$ Tris buffer (pH 7.4) at 25°C. [P–SH] = $10^{-5}$ $M$; [R–S–S–R] = $2 \cdot 10^{-4}$ $M$.

| Disulphide | Protein | $k_1$ $(min^{-1})$ | $k_{-1}$ $(min^{-1})$ | $K_{eq} = k_1/k_{-1}$ | Literature $k_1$ $(min^{-1})$ |
|---|---|---|---|---|---|
| I | Hb | 990 ± 50 | 90 ± 10 | 11 | |
| | HSA | * | * | | |
| | BSA | * | * | | 60 000** |
| II | Hb | 50 ± 10 | 5100 ± 200 | 1/102.0 | |
| | HSA | 70 ± 10 | 5200 ± 200 | 1/74.3 | |
| | BSA | 190 ± 10 | 11 000 ± 500 | 1/57.9 | < 300** |
| III | Hb | 340 ± 20 | 4200 ± 200 | 1/12.4 | |
| | HSA | 370 ± 20 | 3900 ± 200 | 1/10.5 | |
| | BSA | 940 ± 50 | 7400 ± 300 | 1/7.9 | |
| IV | Hb | 4100 ± 200 | 190 ± 10 | 21.6 | |
| | HSA | 2100 ± 100 | 100 ± 10 | 21.0 | |
| | BSA | 3500 ± 200 | 160 ± 10 | 21.9 | 4200** |
| V | Hb | 790 ± 50 | 230 ± 20 | 3.4 | |
| | HSA | 610 ± 50 | 130 ± 10 | 4.7 | |
| | BSA | 1300 ± 100 | 150 ± 10 | 8.7 | |

\* For the disulphide I, kinetic constants $k_1$ were determined by the second competition method (cf., Table II).
\*\* Kinetic constants reported in the literature[22] were obtained in 0.005 $M$ Tris buffer (pH 7.8) with an ionic strength of 0.02 at 25°C.

netic fixation curves in Fig. 6, obtained from the present measurements, are in good agreement with those published previously[1].

From the values of the different kinetic constants it appears that BSA is the most reactive of the proteins studied, and that Hb and HSA have very similar kinetic parameters for most disulphides. However, a significant difference appears for IV, for which the reaction with Hb is of a significantly higher order than for HSA.

*Comparison of electrophoresis and HPLC of modified Hb*

In order to compare HPLC and electrophoresis in the disulphide fixation of proteins, we compared the HPLC of different modified haemoglobins with our previous results[1]. The results are shown in Fig. 7. All experiments were performed after a time long enough to consider that equilibrium was reached. In some instances the Hb modification was complete, as shown by both electrophoresis and HPLC.

When the residue added to the SH function is negatively charged, an increase in the chromatographic retention compared with that of unmodified Hb was ob-

TABLE II

KINETIC CONSTANTS DETERMINED BY THE SECOND COMPETITION METHOD (cf., EQN. 11) FOR THREE DISULPHIDES AND HAEMOGLOBIN, HUMAN SERUM ALBUMIN AND BOVINE SERUM ALBUMIN

Disulphides used: I, cystamine; VI, 2,2'-dithiobis(pyridine); VII, 4,4'-dithiobis(pyridine). Proteins used: Hb, haemoglobin; HSA, human serum albumin; BSA, bovine serum albumin. The kinetics of disulphide binding to protein were determined in 0.02 $M$ Tris buffer (pH 7.4) at 25°C. [P–SH] = $10^{-5}$ $M$; [R–S–S–R] = $2 \cdot 10^{-4}$ $M$.

| Disulphide | Protein | $k_1$ $(min^{-1})$ | Literature $k_1$ $(min^{-1})$ |
|---|---|---|---|
| I | Hb | 940 ± 100 | |
| | HSA | 4500 ± 500 | |
| | BSA | 29 000 ± 2000 | 60 000[*] |
| VI | Hb | 4900 ± 500 | |
| | HSA | 7900 ± 800 | 11 000[**] |
| | BSA | 34 000 ± 3000 | 32 000[**] |
| VII | Hb | 7600 ± 800 | |
| | HSA | 12 000 ± 1000 | |
| | BSA | 49 000 ± 5000 | |

   [*] Kinetic constants reported in the literature[22] were obtained in 0.005 $M$ Tris buffer (pH 7.8) with an ionic strength of 0.02 at 25°C.
   [**] Kinetic constants reported in the literature[21] were obtained in 0.1 $M$ phosphate buffer (pH 7.4) at room temperature.

served. The binding of a positively charged residue (cysteamine) to Hb decreased its retention time and allowed a good separation from unmodified Hb. When the reagent produced no charge at pH 8.0, the retention time of the Hb was not changed.

It must be pointed out that the difference between the retention of modified and unmodified Hb can be changed by varying the chromatographic elution conditions (e.g., by modifying the gradient slope). It appears that ion-exchange chromatography gave a separation based essentially on the protein charge, as the fixation of one negatively charged moiety on Hb produced the same retention of the protein regardless of the different structures of the added compounds. From previous electrophoretic titrations of Hb $A_0$[16] at pH 8.0, the charge on the protein is estimated to be 7 ± 2[25]. By adding a negative charge by reaction with Cys $\beta$ 93 SH, we modified the overall charge by about 15%. This change is sufficient to obtain a good separation by ion-exchange chromatography.

Considering the electrophoretic results in Fig. 7, it is observed that the separation between modified and unmodified Hb is complete when the added reagent bears a negative charge. The electrophoretic separation is large enough to allow quantitative determination by slab densitometry. With the uncharged thiopyridine moiety, anion-exchange chromatography does not allow separation, as expected, whereas the electrophoretic mobility of the modified Hb changes slightly, surprisingly in the same sense as with a negatively charged reagent.

Despite the similar performances of electrophoresis and HPLC in the separation of reacted and unreacted proteins, electrophoresis cannot be used for studying
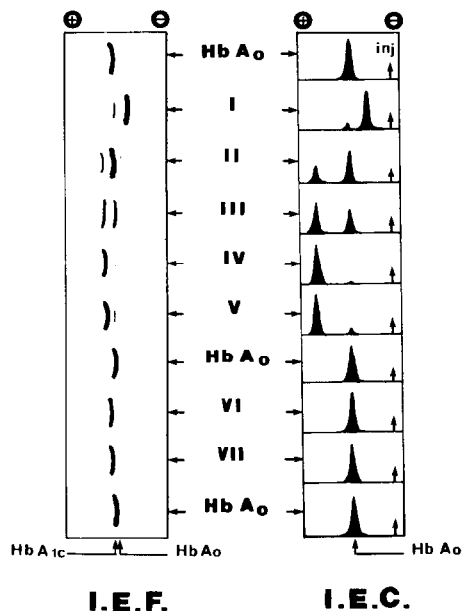
Fig. 7. Comparison of haemoglobin-modified separation by isoelectric focusing (IEF) and ion-exchange chromatography (IEC). All experiments were performed after a time long enough to consider that equilibrium was reached. Incubations were performed at 25°C in 0.02 $M$ Tris buffer (pH 7.4). [Hb $A_0$] = $10^{-5}$ $M$; [R–S–S–R] = $2 \cdot 10^{-4}$ $M$. I, Hb $A_0$ modified by cystamine; II, Hb $A_0$ modified by oxidized glutathione; III, Hb $A_0$ modified by 2,2'-dithiobis(benzoic acid); IV, Hb $A_0$ modified by 5,5'-dithiobis(2-nitrobenzoic acid); V, Hb $A_0$ modified by 6,6'-dithiobis(nicotinic acid); VI, Hb $A_0$ modified by 2,2'-dithiobis(pyridine); VII, Hb $A_0$ modified by 4,4'-dithiobis(pyridine).

the reaction kinetics, especially for short reaction times. In fact, it is unlikely that the reaction between protein and reagent stops after the deposition on the electrophoretic slab. This technique is therefore applicable only to the study of slow reactions. In contrast, owing to the speed of the separation by HPLC, the excess reagent cannot react further with the protein and the extent of the reaction can be measured for times as short as 2 min. This point is of interest in connection with the use of a marker, as short reaction times (2–5 min) are necessary. Owing to the rapidity of sample transfer and injection (about 10 s), the error in the reaction time is small.

CONCLUSION

HPLC on Synchropak AX-300 was applied to the determination of kinetic parameters for the fixation of several disulphides to some proteins. The HPLC method is independent of the spectra of the released thiol (in contrast to spectrophotometry) and is especially well suited when the disulphide bears an electrically charged group. By using a competition method, we extended the measurement to uncharged disulphides. HPLC appears to be superior to electrophoresis in permitting the determination of the kinetic characteristics of rapid reactions involving proteins.

The method was tested with charged and uncharged disulphides and led to kinetic values very close to those previously obtained. Similar determinations with

various disulphides and other thiol reagents are at present under study in our laboratory.

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