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ISOLATION OF HUMAN HAEMOPEXIN BY BIOAFFINITY CHROMATOGRAPHY ON HAEME-SEPHAROSE

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SUMMARY

A preparative procedure was developed for the isolation of human apo-haemopexin from Cohn fraction IV or blood serum, based on bioaffinity chromatography on haeme-Sepharose. The isolation is carried out in the pH range 4–8; hence the possibility of degradation of the carbohydrate moiety of the glycoprotein in the acidic media used in other isolation procedures is decreased. Owing to the conditions of the separation and the good stability of the affinity support, the column can be used repeatedly for long periods without a significant loss of binding capacity. The reversibility of the conformational changes that haemopexin undergoes in acidic media was examined by hydrophobic chromatography. The original hydrophobic characteristics were restored only approximately 48 h after haemopexin had been brought into a neutral medium.

INTRODUCTION

Haemopexin is a single-chain protein with a relative molecular weight of *ca.* 57,000[1], of which roughly 20% represents the carbohydrate moiety[2]. Haemopexin belongs to specific blood transport proteins whose concentration in the blood serum is 0.6–1.0 g/l[3], and binds very strongly the haeme released in the bloodstream during the degradation processes. Its physiological function and physico-chemical characteristics have been reported earlier[4,5].

Haemopexin has been isolated from blood serum by several procedures, making use of selective precipitation and ion-exchange chromatography[6–13], in yields below 20%. Bioaffinity chromatography, based on the binding of the carbohydrate moiety of the molecule to wheat germ lectin-Sepharose[14] or on the interaction of apo-haemopexin with the haeme bonded to Sephadex G-100[15], Bio-Gel P200[16] or Sepharose 4B[17], has also been used for the isolation of a haemopexin. The aim of this study was to improve the isolation by bioaffinity chromatography on a support with immobilized haeme[16,17], in order to avoid the action of strongly acidic media on haemopexin, and by increasing the lifetime of the column by employing a novel mode of bonding the haeme to the affinity support.

EXPERIMENTAL

Sepharose 4B, Sepharose CL-4B and Sephadex G-10, G-100, and G-25 Fine were obtained from Pharmacia (Uppsala, Sweden), and Spheron P300 (63–100 μm) from Lachema (Brno, Czechoslovakia). Octyl-Sepharose was prepared from Sepharose CL-4B according to Hjertén *et al.*[18]. Polyamide layer sheets were purchased from BDH (Poole, Great Britain). Acrylamide, N,N′-methylenebisacrylamide and sodium dodecyl sulphate were purchased from Koch-Light (Colnbrook, Great Britain), Tris [N-tris(hydroxymethyl)aminomethane] and 1-dimethylaminonaphthalene-5-sulphonyl chloride from Serva (Heidelberg, G.F.R.) and Amido black 10B from Merck (Darmstadt, G.F.R.). Transferrin was a B-grade product of Calbiochem (San Diego, CA, U.S.A.). Horse antihuman sera (anti-haemopexin, anti-albumin, anti-transferrin and anti-human serum) were obtained from Sevac (Prague, Czechoslovakia). A standard preparation of Cohn fraction IV in lyophilized form and human serum albumin (salt-free, 98%) were obtained from Imuna (Šarišské Michalany. Rivanol (2-ethoxy-6,9-diaminoacridine lactate) was purchased from Zdravotnické Zásobování (Prague, Czechoslovakia), epichlorohydrine (pure) from Jenapharm (Jena, G.D.R.), sodium borohydride from Metallgesellschaft (Frankfurt/Main, G.F.R.), 2-mercaptoethanol (pure) from Loba Chemie (Vienna, Austria), barium hydroxide (analytical-reagent grade) from Merck and 1,4-dibromobutane (pure) from Fluka. Dicyclohexylcarbodiimide was prepared as described elsewhere[18]. The remaining chemicals were analytical-reagent grade materials from Lachema.

Preparation of haeme-Sepharose

Haemin (protoporphyrin IX chloride) was prepared by the method described elsewhere[19] but using a volume of acetone three times greater than that reported by the authors. The product was recrystallized twice from pyridine–acetic acid–chloroform[19]. Haeme-Sepharose was prepared according to the scheme shown in Fig. 1. SH-Sepharose was prepared by a procedure described elsewhere[20]. The content of SO_3H groups in our preparation, determined by acidimetric titration prior to the reduction, was 0.4 mmol per 100 ml. This material can be replaced with a commercial preparation of thiopropyl-Sepharose 4B (Pharmacia).

Preparation of 8-amino-2-hydroxy-4-thiooctyl-Sepharose (V)

Thiopropyl-Sepharose (III) was washed with water, filtered off and transferred into a mixture of 200 ml of ethanol, 70 ml of water, 30 ml of 0.1 *M* ammonium hydrogen carbonate containing 1 m*M* EDTA, and 25 ml of 1,4-dibromobutane. The mixture was stirred thoroughly and allowed to stand for 45 min at 50°C. The slurry was washed with ethanol–water (4:1) and transferred into 250 ml of 10% ammonia solution. The reaction mixture was set aside for 10 h at room temperature and then washed thoroughly with water.

Binding of haeme

The gel (V, 150 ml) was washed with 250 ml of dimethylformamide (DMF), filtered off, then treated with 300 ml of DMF together with a solution of 50 mg of haemin in 30 ml of DMF and a filtered solution of 3 g of dicyclohexylcarbodiimide in 30 ml of DMF. The reaction mixture was set aside for 15 h at room temperature. The
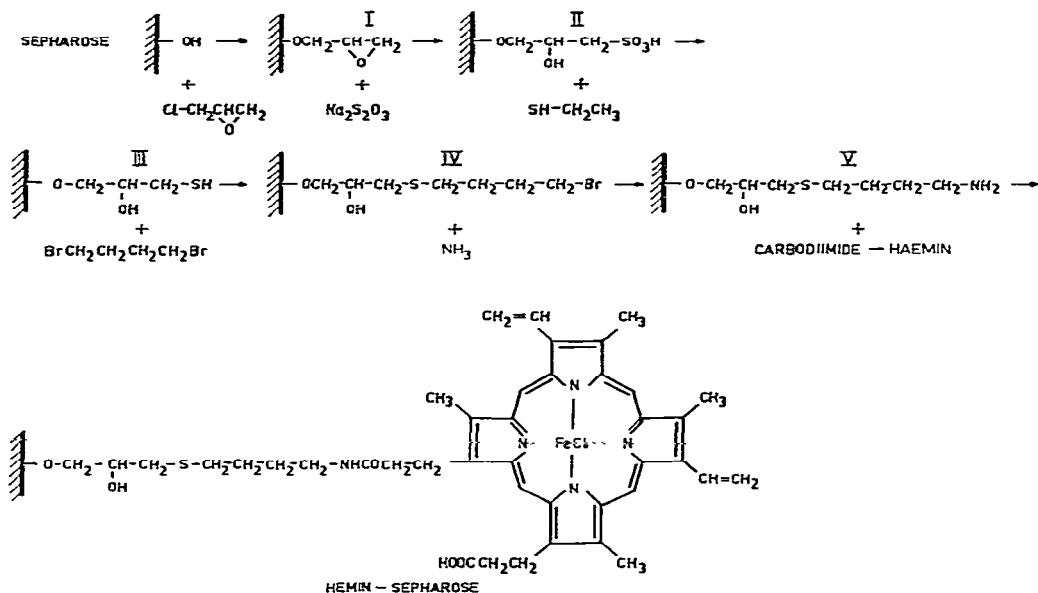
Fig. 1. Scheme of preparation of haeme-Sepharose.

gel was filtered off and the reaction was repeated with 50 mg of haemin dissolved in a mixture of 100 ml of DMF, 50 ml of water and 300 ml of ethanol. The gel was washed with two 200-ml portions of DMF, two 200-ml portions of methanol, water and a saturated solution of sodium tetraborate until the filtrate became only slightly discoloured. The remaining haeme not bound covalently was removed from the column by repeatedly washing it stepwise with several 50-ml portions of 1 % serum albumin, 200-ml portions of 0.05 M Tris–hydrochloric acid buffer (pH 7.0) and 100-ml portions of 0.1 M citrate buffer (pH 4.0) until the red colour of any of the washing solutions had disappeared.

Isolation of haemopexin from Cohn fraction IV

Lyophilized Cohn fraction IV (5 g) was suspended in 50 ml of water and the pH of the turbid solution was adjusted to 8.0 by the addition of 0.5 M sodium hydroxide solution. The solution was cooled to 4°C and 75 ml of pre-cooled 1.68 % rivanol solution (pH 8.0) was added in portions. The albumin precipitate was removed by filtration through a cotton-wool pad. The filtrate was treated with sodium chloride added to a final concentration of 5 %. The precipitate of the insoluble rivanol salt was separated by filtration (Schleicher and Schüll filter-paper No. 589) and the traces of rivanol remaining in the solution were removed by the addition of 4 g of Spheron P300. After stirring for 20 min the Spheron was separated by filtration and the pure filtrate was adjusted to pH 7.0 and applied to a column of haeme-Sepharose (20 × 2.5 cm), equilibrated with 0.05 M Tris–hydrochloric acid buffer (pH 7.0) containing 0.2 M sodium chloride. Unretained proteins were washed out with 350 ml of the same buffer. The protein attached was displaced with 0.1 M citrate buffer (pH 4.0); the effluent fractions were assayed by absorbance measurement at 280 nm. The course of the separation is shown in Fig. 2. The fractions pooled were desalted on Sephadex G-
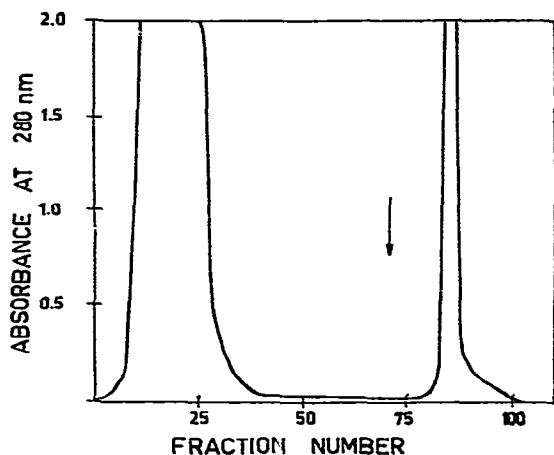
Fig. 2. Affinity chromatography of rivanol supernatant of Cohn fraction IV on haeme-Sepharose.

25 in dilute ammonia solution (pH 8); the yield was 55 mg of the lyophilized preparation.

Isolation of haemopexin from blood serum

The pooled blood serum obtained from donors (200 ml) was adjusted to pH 8.0 and albumin was precipitated by the addition of 1.68% rivanol solution. The conditions of the experiment were identical with those described above for the treatment of Cohn fraction IV as starting material. The volume of serum used (200 ml) afforded 124 mg of the lyophilized preparation (yield about 85%).

The preparations obtained were of high purity and contained a negligible amount of a mixture of immunoglobulins. As described by other workers[16,17], this mixture can be removed by ion-exchange chromatography. Wherever necessary this removal was effected on a small DEAE-cellulose column (6 × 0.8 cm), equilibrated with 0.01 M Tris–hydrochloric acid buffer (pH 7.0). When a sample solution in the same buffer was applied, the mixture of immunoglobulins was not retained and was washed from the column. Haemopexin was displaced as a sharp peak by the same buffer containing 0.4 M sodium chloride. The total yield of this purification procedure was 70% based on the original serum.

Analytical procedures and evaluation of the individual preparations

The composition of the pooled fractions was checked by discontinuous electrophoresis in 7.5% polyacrylamide gel in Tris–glycine buffer (pH 8.3)[21] and by discontinuous electrophoresis in 10% polyacrylamide gel of reduced samples in the presence of 1% sodium dodecyl sulphate in 0.1 M phosphate buffer (pH 7.2)[22]. Unlike the former electrophoresis, under these conditions haemopexin can be separated from transferrin. The protein bands were stained with Amido Black 10B or with Coomassie Brilliant Blue G-250 (the latter procedure). The purity of the preparations obtained was checked by immunoelectrophoresis[23] and by capillary isotacho-.phoresis using the apparatus developed in this Institute[24], equipped with a PTFE capillary of I.D. 0.45 mm and length 45 cm.
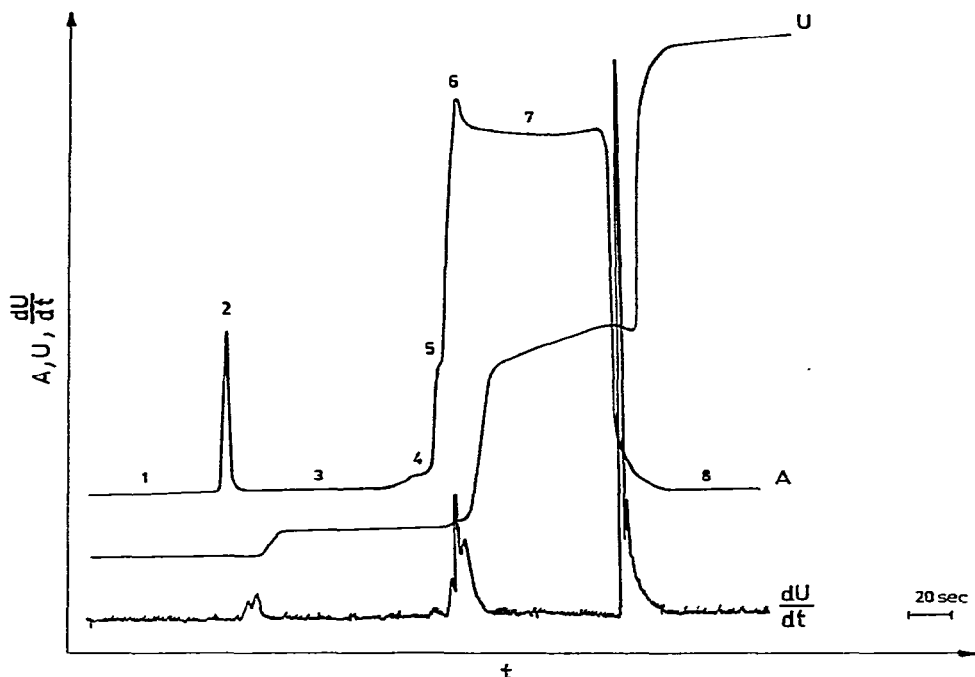
Fig. 3. Analysis of haemopexin (HX) by capillary isotachophoresis. 1 = Chlorides; 2, 4, 5, 6 = unidentified contaminants (6 = probably a dimer of HX); 3 = carbonates; 7 = HX; 8 = Gly$^-$. A = absorbance at 254 nm; U = potential gradient; dU/dT = derivation of potential gradient; t = time. Conditions of separation: leading electrolyte, 0.01 M hydrochloric acid–0.02 M Tris; leading anion, Cl$^-$; counter ion, Tris$^+$; pH, 8.3; additive, poly(vinyl alcohol) (Mowiol; Merck), 0.02% (w/v); terminating electrolyte, 0.01 M glycine. pH 10.2, adjusted with saturated barium hydroxide solution; the amount of sample applied was 62.5 μg in 5 μl of water. The analysis was carried out at a constant temperature of 15°C under a constant current whose level was changed stepwise in the course of the separation as follows: 5 min. 50 μA; 20 min, 80 μA; 5 min. 50 μA; 20 μA during the detection.

The zones were detected by a contact universal potential gradient detector and by an absorption detector at 254 nm. Additional details are given in Fig. 3. The N-terminal amino acid residues were determined as 1-dimethylaminonaphthalene-5-sulphonyl (DANS) derivatives[25] by thin-layer chromatography on polyamide layer sheets[26]. The spectrophotometric measurements were carried out with a Zeiss PMQ II spectrophotometer.

Chromatographic studies of haemopexin renaturation

Haeme-haemopexin (0.7 mol of haeme per mole of protein) was dissolved in water (0.77 mg/ml) and the pH was adjusted to 2.2 with 0.5 M hydrochloric acid. After haemopexin had been exposed to the acidic conditions for 150 min at 20°C, the pH of the solution was increased to 8.0 with 0.5 M sodium hydroxide solution. At pre-determined time intervals 75-μl samples of the solution were applied to a 130 × 8 mm I.D. column of octyl-Sepharose CL-4B. The elution was effected with 0.05 M phosphate buffer (pH 8.0) containing 0.15 M sodium chloride. The chromatographic equipment has been described elsewhere[27].

RESULTS AND DISCUSSION

The predominant part of the proteins in the starting material for haemopexin isolation, in either Cohn fraction IV or blood serum, consists of albumin. Like other workers[17], we employed precipitation with rivanol as the first isolation step. The removal of the last traces of rivanol from the supernatant was facilitated by the addition of Spheron P300, replacing filtration through a Sephadex G-25 column. The composition of the starting material after the rivanol fractionation is shown in Fig. 4. During affinity chromatography (Fig. 2) the haemopexin adsorbed is liberated under relatively mild conditions (pH 4.0) and the danger of deterioration of the sugar moiety of haemopexin in acidic media is thus diminished. No additional protein-containing material was released by elution of the column by a buffer of pH 2.4[17].
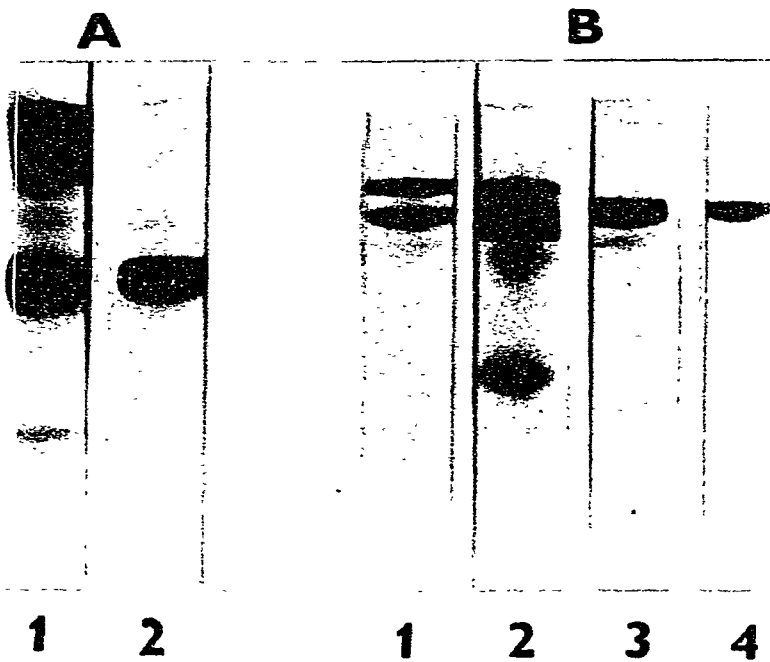

Fig. 4. Discontinuous electrophoresis of the preparations obtained. (A) Electrophoresis at pH 8.3; 1 = rivanol supernatant of Cohn fraction IV; 2 = mixture of hemopexin and transferrin (fraction "E" obtained in earlier work[28]). Haemopexin and transferrin remain unseparated under the conditions of this electrophoretic experiment. (B) Electrophoresis of reduced samples in the presence of 1 % sodium dodecyl sulphate (pH 7.2); 1 = mixture of transferrin (top band) and haemopexin (bottom band; *cf.*, A-2); 2 = rivanol supernatant of blood serum; 3 = preparation of haemopexin after affinity chromatography; 4 = haemopexin purified on DEAE-cellulose.

The modification of the haeme bonding to Sepharose results in good efficiency of the column. As the spacer used did not introduce any electric charges, the protracted tailing of the protein was minimized. Haemopexin was eluted as a sharp peak in a small volume and the exposure of the protein to acidic conditions was short. As the attachment of the haeme to the matrix involved only one bond sensitive to hydrol-

ysis and as mild elution conditions were chosen, the affinity column showed excellent stability: during 1 year of use no significant decrease in its capacity was observed. The advantageous properties of the affinity support can be ascribed to the modified binding of the haeme to the matrix. The binding to Sepharose via the propionyl residue in position 6 or 7 (Fig. 1) obviously does not affect the binding ability of the immobilized haeme. The spacer is sufficiently long and flexible to provide for good exposure of the porphyrin ring necessary for the interaction with haemopexin.

The yield of haemopexin after the precipitation and affinity chromatography was very good, about 85% when haemopexin was isolated from blood serum. The preparation obtained was very pure (Figs. 3–5) and a negligible amount of a mixture of immunoglobulins was detected. This mixture has also been detected by Suttnar et al.[17] in their preparation obtained by affinity chromatography using an affinity support with a different haeme bonding. The total yield of our preparation after purification by chromatography on DEAE-cellulose was about 70%; the use of DEAE-Sepharose CL-6B[17] seems more advantageous. The final preparation did not contain any protein contaminants, as confirmed by the determination of only one N-terminal end group (threonine).
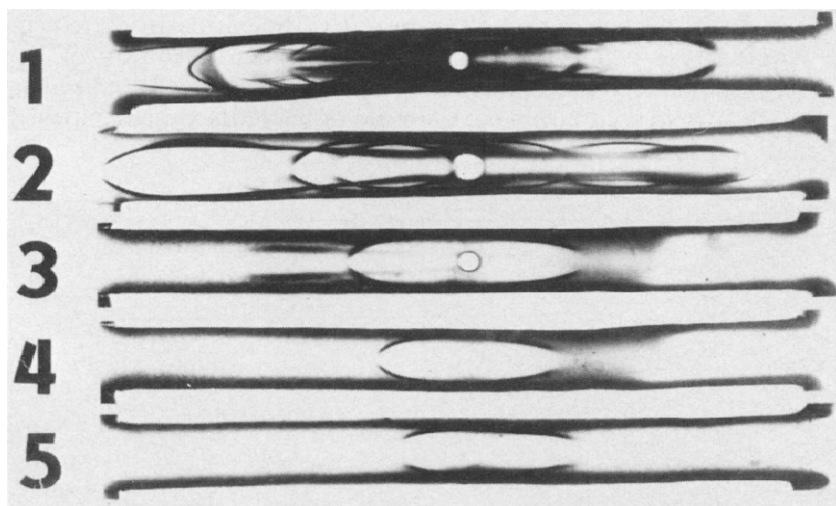

Fig. 5. Immunoelectrophoresis of the individual preparations. 1 = Blood serum; 2 = rivanol supernatant of blood serum; 3 = preparation of haemopexin obtained by affinity chromatography; 4 = haemopexin purified on DEAE-cellulose (all against human serum antiserum); 5 = purified haemopexin (sample 4) against human haemopexin antiserum.

In an earlier study[28] we investigated the isolation of haemopexin from a material containing this protein predominantly as haeme-haemopexin, which cannot be purified by bioaffinity chromatography on supports with immobilized haeme. The remaining content of transferrin could be removed by the procedures used only with difficulty. The liberation of apo-haemopexin from the strong complex with the haeme and the isolation of the free protein is difficult. Evidence has been obtained[29] that this can be approached to a certain extent in the presence of material competing for the

haeme liberated. The most successful has been the procedure which involves disso-
lution of the haeme-haemopexin preparation in 0.1 M glycine–hydrochloric acid
buffer (pH 2.4) and the separation of the haeme liberated by filtration through a
column of Spheron H1000 (Laboratory Instruments Works, Prague, Czechoslo-
vakia). By this procedure the free apo-haemopexin content of the preparations was
considerably enriched and the latter could be isolated in pure form by affinity chro-
matography as described above; the contamination with immunoglobulins was re-
moved by the preceding procedure.

The effect of acidic media on haemopexin has been studied by Hrkal et al.[30].
They concluded that the unfolding of the haemopexin molecule and the dissociation
of the haeme from the binding site are reversible processes and the restoration of the
native conformation proceeds relatively quickly. Our experiments with hydrophobic
chromatography on octyl-Sepharose (Fig. 6) have shown that the reconstitution of
certain amino acid residues in the spatial positions is slower than the transitions
followed in terms of changes of the absorption spectrum in the Soret region. These
changes which take place in acidic media made certain hydrophobic residues ac-
cessible to interaction with the chromatographic support and result in complete hold-
up of haemopexin in the column. The interaction of the hydrophobic residues with
the chromatographic support decreases and the amount of haemopexin eluted in-
creases during the renaturation which takes place after the neutralization of the
solution. A period of approximately 48 h, however, would be necessary for complete
renaturation, indicated by a yield comparable to the yield of haemopexin not exposed
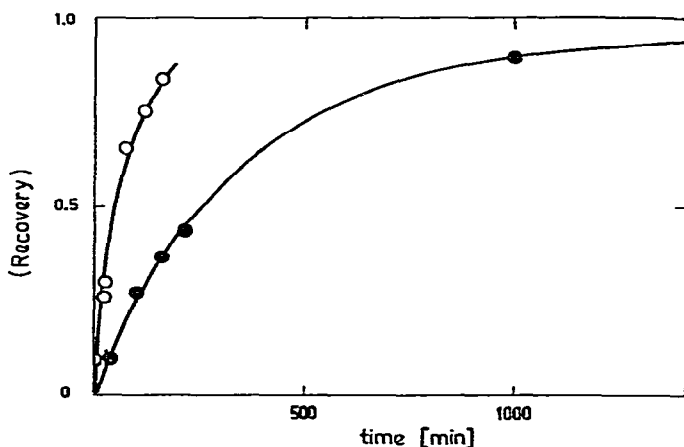to acidic media.

Fig. 6. Renaturation of haemopexin after conversion from acidic to neutral pH. Open symbols: renatur-
ation of haemopexin as a function of time examined spectrophotometrically at 414 nm as the ability to bind
the heme. Recovery $= [A_{414(t)} - A_{414(t=0)}]/[A_{414(t=\infty)} - A_{414(t=0)}]$, where $A_{414(t)}$ is the absorbance at
time t, $A_{414(t=0)}$ the absorbance of the haemopexin sample immediately after conversion to neutral pH and
$A_{414(t=\infty)}$ the absorbance of the unacidified haeme-haemopexin solution; all solutions in 0.05 M Tris–
hydrochloric acid buffer (pH 8.0). Closed symbols: renaturation of haemopexin as a function of time
examined by hydrophobic chromatography. The recovery is represented by the ratio of the eluted acid-
treated to untreated haemopexin.

ACKNOWLEDGEMENTS

REFERENCES

1 V. L. Seery, G. Hathaway and U. Muller-Eberhard, *Arch. Biochem. Biophys.*, 150 (1972) 269.
2 Z. Hrkal and U. Muller-Eberhard, *Biochemistry*, 10 (1971) 1746.
3 H. J. Braun and F. W. Aly, *Klin. Wochenschr.*, 49 (1971) 451.
4 U. Muller-Eberhard and W. T. Morgan, *Ann. N.Y. Acad. Sci.*, 244 (1975) 624.
5 Z. Hrkal, *Biochem. Clin. Bohemoslov.*, 4 (1975) 141.
6 G. Biserte, R. Havez and J. Laturaze, *Pathol. Biol.*, 9 (1961) 1681.
7 H. E. Schultze, K. Heide and H. Haupt, *Naturwissenschaften*, 48 (1961) 696.
8 K. Heide, H. Haupt, K. Störiko and H. E. Schultze, *Clin. Chim. Acta*, 10 (1964) 460.
9 U. Muller-Eberhard and E. C. English, *J. Lab. Clin. Med.*, 70 (1967) 619.
10 Z. Hrkal, Z. Vodrážka and J. Rejnková, *J. Chromatogr.*, 72 (1972) 198.
11 A. Hayem-Lévy and R. Havez, *Clin. Chim. Acta*, 47 (1973) 113.
12 P. Aisen, A. Leibman, D. C. Harris and T. Moss, *J. Biol. Chem.*, 249 (1974) 6824.
13 Y. Plancke, M. Dautreveux and G. Biserte, *FEBS Lett.*, 78 (1977) 291.
14 P. Vratblad and R. Hjorth, *Biochem. J.*, 167 (1977) 759.
15 K. Heide, *Behringwerk-Mitt.*, 46 (1966) 115.
16 J. Suttnar, Z. Hrkal and Z. Vodrážka, *J. Chromatogr.*, 131 (1977) 453.
17 J. Suttnar, Z. Hrkal, Z. Vodrážka and J. Rejnková, *J. Chromatogr.*, 169 (1979) 500.
18 S. Hjertén, J. Rosengren and S. Påhlman, *J. Chromatogr.*, 101 (1974) 281.
19 R. F. Labbe and G. Nishida, *Biochim. Biophys. Acta*, 26 (1957) 437.
20 J. Carlsson, R. Axén and T. Unge, *Eur. J. Biochem.*, 59 (1975) 567.
21 B. J. Davis, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
22 K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406.
23 J. J. Scheidegger, *Int. Arch. Allergy Appl. Immunol.*, 7 (1964) 404.
24 Z. Prusík, J. Štěpánek, K. Ženíšek and V. Kašička, unpublished results.
25 W. R. S. Gray and B. S. Hartley, *Biochem. J.*, 89 (1963) 59p.
26 B. S. Hartley, *Biochem. J.*, 119 (1970) 805.
27 P. Štrop, F. Mikeš and Z. Chytilová, *J. Chromatogr.*, 156 (1978) 239.
28 L. Morávek, J. Borvák, K. Grüner, B. Meloun, P. Štrop, Z. Hrkal and M. Pokorný, *Collect. Czech. Chem. Commun.*, in press.
29 P. Štrop, J. Borvák and L. Morávek, unpublished results.
30 Z. Hrkal, M. Kodíček and Z. Vodrážka, *Ann. Clin. Res.*, 8, supp. 17 (1976) 239.