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# Efficient method of haemoglobin F enrichment in adult haemolysate for determination of the γ-chain isoforms by high-performance liquid chromatography

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ABSTRACT

An alkali denaturation procedure was developed for the efficient enrichment of haemoglobin F (Hb F) in human adult haemolysate. In contrast to the previous procedures, the method is readily applicable to small amounts of blood containing less than 1% of Hb F, providing reproducibly a sufficient level of Hb F (at least 11%) to allow an accurate high-performance liquid chromatographic determination of the three γ-chain isoforms Further enrichment was possible by combination with CM-Sephadex chromatography

## INTRODUCTION

The phenomenon of haemoglobin (Hb) F to A switching is an excellent model for research on the developmental control of gene expression in mammals. Schroeder *et al* [1] first reported that there were two isoforms in the γ-chain of Hb F, namely $^{G}\gamma$ (75Ile, 136Gly) and $^{A}\gamma$ (75Ile, 136Ala), and also that the $^{G}\gamma/^{A}\gamma$ ratio underwent a definite postnatal change. Later, the $^{A}\gamma$ chain was found to have two isoforms, $^{A}\gamma^{I}$ (75Ile, 136Ala) and $^{A}\gamma^{T}$ (75Thr, 136 Ala) [2].

The isolation and determination of the γ-chain isoforms were much facilitated by the advent of high-performance liquid chromatography (HPLC) [3–5]. However, the difficulty of obtaining an adult haemolysate containing a sufficient amount of Hb F for reliable determination of isoforms has not yet been overcome. So far two procedures for this purpose, one using alkaline denaturation [6] and the other anion-exchange chromatography [7], have been reported. Bakioglu *et al.* [8] recently compared these two methods and concluded that "it seems desirable to develop additional micro methods which will allow an improved separation of Hb F .." in view of the finding that "the two approaches do not always provide a Hb solution with an adequate amount of Hb F to allow an accurate quantitation of the three types of γ-chain", especially when applied to normal blood samples with low levels of Hb F (< 1%).

Here we describe a novel version of the alkali denaturation procedure, provid-

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ing a reliable and higher enrichment of Hb F, which has been successfully used in this laboratory for more than a decade [9,10]

## EXPERIMENTAL

### *Blood samples*

Blood was obtained from normal adults by venipuncture and heparinized. In some experiments red cells stored in CPD medium, supplied from a local blood bank, were also used. After washing with saline and lysing with water and toluene followed by refrigerated centrifugation (19 000 *g*, 20 min) the resulting haemolysate was adjusted to an Hb concentration of 10%, flushed with carbon monoxide and stored on ice.

### *Enrichment of Hb F from adult haemolysate*

A one tenth volume of 5 *M* NaOH was added to the freshly prepared haemolysate with continuous stirring at 20°C. The final concentration of NaOH was 0.45 *M*. To the mixture, after standing for 2 min, were added 1.95 volumes of a 50% saturated ammonium sulphate solution containing HCl. The solution was prepared by dissolving 380 g of ammonium sulphate and 23.7 ml of concentrated HCl in 1 l of deionized water. On adding the sulphate solution the mixture was neutralized and alkali-denatured protein (mostly Hb A) was precipitated. In the latter half of this study we used oxygenated, instead of carboxy, haemolysate in which the duration of alkali exposure was reduced to 30 s (see below). After refrigerated centrifugation (19 000 *g*, 20 min) the supernatant was dialysed against several changes of carbon monoxide-saturated deionized water until sulphate ion could not be detected in the dialysis medium. Although the resulting samples were usually found to contain more than 10% Hb F (Table 1B), we attempted further enrichment of the protein by CM-Sephadex chromatography [11]: the supernatant, after concentration by ultrafiltration, was dialysed against carbon monoxide-saturated 0.05 *M* phosphate buffer (pH 6.0) containing 40 m*M* NaCl, loaded onto a CM-Sephadex column equilibrated with the same buffer, the size of which was varied depending on the sample size, and developed with a linear salt gradient from the above buffer to 0.05 *M* phosphate (pH 6.0) containing 160 m*M* NaCl. The first-eluting peak (Hb F) after a small void fraction was pooled, dialysed against carbon monoxide-saturated deionized water and concentrated. Care was taken always to ensure carbon monoxide saturation of the samples. All the chromatographic procedures were conducted in the cold (4°C).

### *Determination of the three isoforms of the γ-chain*

A reversed-phase HPLC procedure, originally reported by Huisman *et al.* [12], was generally applied with a Shimadzu HPLC LC-4A instrument. The samples prepared as above were exposed with gentle shaking to an oxygen stream and fluorescent light in the cold, by means of which carbon monoxide bound to Hb

was removed. Usually 250 μg (2.5%, 10 μl) oxy Hb, filtered through a Millipore filter (SJHVOO4NS, pore size 0.45 μm) immediately prior to the analysis, were applied to a Waters Assoc. μBondapak $C_{18}$ column (250 mm × 4.6 mm I.D.) equilibrated with solvent A–B (20:80) for 30 min (see below). The column was equipped with a guard column (Shimadzu Permaphase ODS). The solvents used were (A) acetonitrile–methanol–(49 m*M* $KH_2PO_4$–5.4 m*M* $H_3PO_4$, pH 2.84) (53:5:45, v/v), (B) acetonitrile–methanol–(49 m*M* $KH_2PO_4$–5.4 m*M* $H_3PO_4$, pH 2.84) (38:9.5:52.5, v/v) and (C) acetonitrile–water (50:50). The column, equilibrated with solvent A–B (20:80) for 30 min, was eluted isocratically for the first 10 min, and then with a linear gradient to solvent A–B (63:37) in 134 min. The flow-rate was 1.5 ml/min and the absorbance of the eluate was monitored at 220 nm. After the 144-min elution was completed, the column was purged with solvent A–B (99:1) for 15 min and re-equilibrated with the initial solvent mixture for 30 min for the next use.

At the end of each day's operation, the column was equilibrated with solvent C after the purge. The percentages of the three types of the γ-chain were calculated with a Shimadzu Chromatopac C-R1B data processor which was connected with the chromatograph.

*Determination of Hb F*

The alkali denaturation procedure of Betke *et al.* [13] was used to determine the Hb F content in adult blood, which was usually less than 1%. Hb F contents in the samples used for HPLC analyses were calculated on the basis of β and γ%, which were known from the HPLC results.

All chemicals were of analytical-reagent grade and were supplied by Nacalai Tesque (Kyoto)

## RESULTS AND DISCUSSION

*Enrichment of Hb F in adult haemolysate*

Table IA shows the enrichment by the alkali denaturation–salting-out procedure with CM-Sephadex chromatography.

The average Hb F concentrations in the starting and final samples were 0.8% (0.3–1.4%) and 74.9% (45.0–96.5%), respectively, thus providing an average enrichment of 108-fold (68–295). It should be noted that the final sample contained at least 45% of Hb F. This result contrasts with the previous procedures [6,7] which were reported not to provide always an Hb solution with a sufficient amount of Hb F to allow an accurate HPLC determination of the γ-chain isoforms [8]. Essentially the same result can be obtained by the procedure without the chromatography, and the final Hb F concentration was at least 11.0%, also sufficient for an accurate determination (Table IB)

TABLE I

ENRICHMENT OF Hb F IN NORMAL ADULT HAEMOLYSATE BY THE ALKALI DENATURATION-SALTING-OUT PROCEDURE WITH OR WITHOUT CM-SEPHADEX CHROMATOGRAPHY

Starting volume of sample 5–10 ml. Values are means ± S.D. Figures in parentheses show ranges of variation

| Enrichment method | | Hb F (%) | | Enrichment ratio[c] |
|---|---|---|---|---|
| | | Initial[a] | Final[b] | |
| (A) | With CM-Sephadex chromatography ($n$ = 25) | 0.75 ± 0.25 (0.3–1.4) | 74.9 ± 15.5 (45.0–96.5) | 108 ± 46 (68–295) |
| (B) | Without CM-Sephadex chromatography ($n$ = 7) | 0.44 ± 0.17 (0.2–0.7) | 31.0 ± 13.1 (11.0–48.9) | 97 ± 55 (26–244) |

[a] By Betke *et al.* [13].
[b] Estimated from the HPLC result.
[c] Final Hb F/initial Hb F.

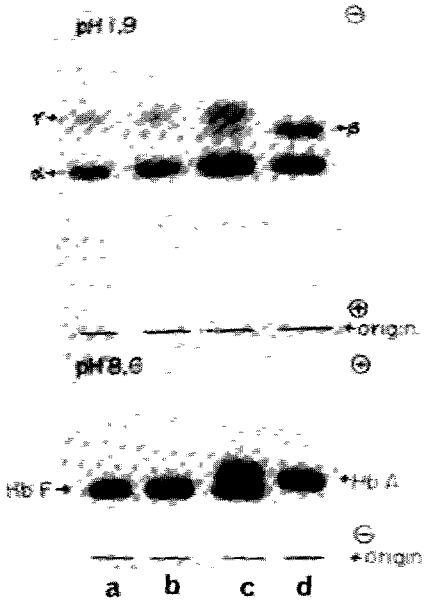


Fig. 1. Enrichment of Hb F in normal adult haemolysate as shown by starch gel electrophoresis. Top: formate buffer system (pH 1.9), in which Hbs were split into the constituent subunits. Bottom: 0.04 *M* Tris–EDTA–borate buffer system (pH 8.6). Amido Black 10B stain. (a) Cord haemolysate, (b) adult haemolysate treated by alkali denaturation–salting-out–CM-Sephadex chromatography, (c) adult haemolysate treated by alkali denaturation–salting-out, (d) adult heamolysate.

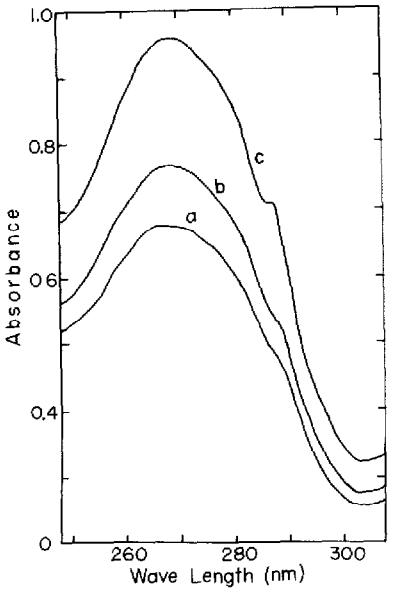


Fig 2 Enrichment of Hb F in normal adult haemolysate as shown by ultraviolet spectrophotometry 0 1 *M* Potassium phosphate buffer (pH 7.0) (a) Adult haemolysate. (b) Hb $A_0$ isolated from adult haemolysate. (c) Hb F-enriched adult haemolysate (alkali denaturation–salting-out)

*Electrophoretic and spectrophotometric characterization of Hb F isolated from adult haemolysate*

Satisfactory enrichment of Hb F from adult haemolysate by the present procedure was shown by starch gel electrophoresis (Fig. 1) and also by ultraviolet spectrophotometry in which a well resolved fine structure band around 290 nm, a feature characteristic of the $\gamma$ subunit [14], could be clearly observed for the adult Hb F (Fig. 2).

*Effect of starting volume of haemolysate samples*

Fig. 3 shows the HPLC profiles of an adult haemolysate as treated by the present procedure with or without CM-Sephadex chromatography but with different starting volumes. The results with two different samples of widely varied starting volumes (1–50 ml) are summarized in Table II.

Neither variation of the starting volume nor additional application of chromatography resulted in any modification of the results, although the final Hb F content was considerably increased by the use of CM-Sephadex chromatography.

*Effect of duration of exposure to alkali*

Table III shows the compositions of the $\gamma$-chain isoforms in adult haemolysates which were exposed to alkali for various periods of time. It is evident that

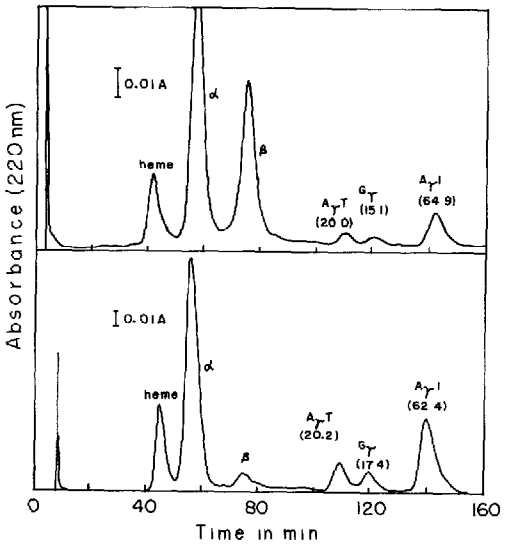


Fig 3 HPLC profiles of Hb F-enriched adult haemolysate Top 1 ml of haemolysate (10% Hb) treated by the alkali denaturation–salting-out procedure, providing a sample with 24 5% of Hb F. Bottom: 50 ml of the same haemolysate treated by alkali denaturation–salting-out–CM- Sephadex chromatography, providing a sample with 89.9% of Hb F $\mu$Bondapak $C_{18}$ (10 $\mu$m) column (250 mm × 4 6 mm I D.). Absorbance was monitored at 220 nm See text for the details

TABLE II

PERCENTAGES OF THE $\gamma$-CHAIN ISOFORMS IN Hb F-ENRICHED NORMAL ADULT HAEMOLYSATE

No dependence on the starting volume of haemolysate

| Sample I | | | Sample II[a] | | | |
|---|---|---|---|---|---|---|
| Starting volume (ml) | $^{G}\gamma$ (%) | $^{A}\gamma$ (%) | Starting volume (ml) | $^{A}\gamma T$ (%) | $^{G}\gamma$ (%) | $^{A}\gamma I$ (%) |
| 50 | 52 5 | 47 5 | 50 | 20 4 | 17 3 | 62 3 |
| 10 | 51 9 | 48 2 | 10 | 20 8 | 15 3 | 63 9 |
| 5 | 54 9 | 45 1 | 5 | 20 3 | 16.0 | 63 7 |
| 2 | 52 6 | 47 4 | 2 | 20.3 | 15 7 | 64 0 |
| 1[b] | 56 9 | 43 1 | 1 | 20 0 | 13 9 | 66 2 |
| 50–CMS[c] | 51 9 | 48 2 | 50–CMS[c] | 20 6 | 17 6 | 61.8 |

[a] Sediments were washed with 1 ml of 50% saturated ammonium sulphate per 1 ml of sample
[b] Sediment was washed once with 50% saturated ammonium sulphate
[c] Further enriched by CM-Sephadex chromatography

TABLE III

PERCENTAGES OF THE THREE γ-CHAIN ISOFORMS IN Hb F-ENRICHED NORMAL ADULT HAEMOLYSATE

No dependence on the duration of exposure of haemolysate to alkali.

| Sample volume (ml) | Duration (s) | $^{A}\gamma^{T}$ (%) | $^{G}\gamma$ (%) | $^{A}\gamma^{I}$ (%) |
|---|---|---|---|---|
| 10 | 30 | 26 1 | 17 3 | 56 6 |
| 10 | 60 | 26 1 | 19 6 | 54.3 |
| 10 | 90 | 25 4 | 19.2 | 55 4 |
| 10 | 120 | 25 6 | 18 8 | 55 6 |
| 10 | 180 | 24 9 | 19 0 | 56 1 |
| 220 | 60 | 26 7 | 18 7 | 54.6 |

duration of the exposure, at least within 3 min, does not influence the results. In subsequent investigations with the carboxy form we used 2 min for alkali denaturation.

*Effect of ligand form of haemolysate samples*

Table IV compares the results obtained with oxy and carboxy forms of two haemolysate samples Use of the oxy form provided a higher enrichment of Hb F (two-fold or more), and the compositions of the γ-chain were essentially the same as those in the carboxy form. It should be also noted that exposure for 30 s gave rise to a sufficient enrichement of Hb F. In view of these results, we used ox-

TABLE IV

PERCENTAGES OF THE γ-CHAIN ISOFORMS IN NORMAL ADULT HAEMOLYSATE IN OXY AND CARBOXY FORM

Sample volume 10 ml Sediments were washed with 50% saturated ammonium sulphate.

| Sample | Duration (s) | Hb F (%) | | $^{A}\gamma^{T}$ (%) | $^{G}\gamma$ (%) | $^{A}\gamma^{I}$ (%) |
|---|---|---|---|---|---|---|
| | | Initial | Final | | | |
| I-oxy | 30 | 0 6 | 79.2 | 27 9 | 17.9 | 54 2 |
| I-oxy | 45 | 0 6 | 86 3 | 25 9 | 18.3 | 55.8 |
| I-oxy | 60 | 0 6 | 80 8 | 24 8 | 18 1 | 57 1 |
| I-carboxy | 120 | 0 6 | 44 2 | 23 8 | 18 7 | 57 4 |
| II-oxy | 30 | 0 4 | 38 1 | 80.3 | 19 7 | – |
| II-oxy | 45 | 0 4 | 59 8 | 81 0 | 19 0 | – |
| II-oxy | 60 | 0 4 | 53 8 | 80 1 | 19 9 | – |
| II-carboxy | 120 | 0.4 | 11 9 | 80 2 | 19 8 | – |

ygenated haemolysates after 30-s alkaline denaturation as the sample for the HPLC analyses in latter half of the study. This modification was found to give a better result especially when the sample volume was limited

One of the problems with Hb F enrichment by an alkali denaturation procedure might be a loss of $\gamma$-chains and/or the formation of modified chains with an altered HPLC behaviour. It is true that a loss of Hb F together with Hb A occurs (Fig. 3). The critical point, however, is whether preferential loss of any one of the three $\gamma$-chains occurs. This was not the case in view of the results in Tables II and III, which show that the isoform composition of the same sample does not change under different conditions of the denaturation. The possibility of chain modification such as modified $^{G}\gamma$ chains being eluted at the $^{A}\gamma^{T}$ position is also inconceivable, as our unpublished results for $^{G}\gamma\%$ of the enriched samples determined by the classical $\gamma$-CB3 procedure of Schroeder *et al.* [1] ($27 \pm 18\%$, $n = 21$) was in fairly good agreement with that obtained by the HPLC procedure ($25.8 \pm 16.8\%$), the values being typical for adults [1]. Two other findings should be noted First, $^{A}\gamma^{T}$ gene frequency, calculated from our results obtained for 206 adult samples by the present procedure, is 0.138, which agrees very well with the value of 0.141 for 259 cord samples The value for newborns, in turn, agrees with the recent result (0.138) reported for the newborn population in Osaka district, very near to the location of the present observations [15]. Second, oxygen equilibrium properties such as oxygen affinity ($P_{50}$), the Bohr effect and the 2,3-diphosphoglycerate effect hardly differ in adult Hb F isolated by the present method and Hb F derived by cation-exchange chromatography [11] from cord blood (data not shown).

So far two methods have been used to enrich Hb F in adult haemolysate with low levels of Hb F ($< 1\%$), namely an alkali denaturation–salting-out procedure by Tsuchiya *et al.* [6] and an anion-exchange chromatographic procedure by Abraham *et al* [7]. In a recent comparative study of these two methods it was concluded that although the results obtained with the two procedures were mostly comparable to each other, they do not always provide sufficient enrichment of Hb F to permit an accurate HPLC determination of the three isoforms of the $\gamma$-chain [8]. We came to the same conclusion.

The alkali denaturation–salting-out procedure delineated here has been conveniently and successfully used in our laboratory for more than a decade with a reliably high yield of Hb F (Table I) [9,10]. The procedure is easy to perform without expensive equipment or reagents, and several samples can be treated simultaneously, hence it is suitable for routine applications Usually we start with a sample volume of 5 ml or more, if available, for convenience of handling, although smaller volumes of blood also give satisfactory results (Table II). Additional separation of Hb F by CM-Sephadex chromatography is generally unneccessary.

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