# Some observations about the heterogeneity of haemoglobin in aluminum oxide chromatography

The heterogeneity of normal adult haemoglobin and of some abnormal haemoglobins is reported by several workers using different methods, for instance alkali denaturation[1], salting out[2], chromatography on Amberlite IRC 50[3,4], paper electrophoresis[5] and chromatography on $Al_2O_3$[6,7]. Using the $Al_2O_3$ column, VAN FOSSAN[6] succeeded in fractionating Hb of both adult and fetus into three distinct components. As this method seemed suitable for detecting the heterogeneity of Hb some experiments were carried out to check the occurrence in blood of the different components postulated. The results of this research are reported here.

The haemoglobins used (A, F, B and C) have been described elsewhere[8]. The preparation was carried out as reported earlier[9]; but the haemoglobin solutions (containing 10 g per cent of COHb) were never dialysed, except in a few special cases described below. Two samples of aluminium oxide suitable for chromatographic analysis were used, one obtained from Merck and one from the British Drug Houses Inc. (B.D.H.).

The general procedure was similar to those described by VAN FOSSAN[6]: The $Al_2O_3$ was poured into the tube (diameter 0.9 to 2.5 cm × 5 to 20 cm) as a thick aqueous suspension and allowed to settle. Small amounts of COHb (5–50 mg) were put on the column and development was carried out with distilled water. The pH of the eluate fractions collected varied between 9.5 and 10.1. Two red water-mobile bands descended in the column close to each other, while a third one which was immobile at the top was eluted with 0.35% ammonia. The amount of Hb in each fraction was determined with a Beckman spectrophotometer at 5700 A.

The results of the fractionation of various human haemoglobins (A, B, C and Hb from cord blood) are given in Table I. Since a relatively large deviation exists for the amounts of the fractions I and II, it will be clear that no significant differences exist between the four haemoglobins studied. It will be noted that the ratio between the three fractions is quite different from that reported by VAN FOSSAN, who found average values of 48% (I), 3% (II) and 49% (III) for adult Hb. These differences may be explained by differences in the batches of $Al_2O_3$, though when B.D.H. and Merck aluminium oxide was used approximately similar results were obtained (Table I).

The influence of dialysis on the Hb solutions is remarkable. When the COHb solution was dialysed against distilled water at 4° C for 96 hours the amount of the ammonia-mobile fraction decreased from 19% to 8%, while moreover only one water-mobile fraction was observed (Table I). The effect of dialysing the Hb solutions against 0.35% ammonia (45 hours at 4° C and 3 hours against distilled water in order to remove the ammonia) is still greater: only 5% of the total amount of haemoglobin remained at the top of the column (Table I). In some experiments the dialysate was concentrated by freeze-drying and recombined in a threefold equivalent amount with the dialysed Hb solution. The chromatographic separation showed an increase in the amount of the ammonia-mobile fraction (from 5% before to 21% after the recombination),

TABLE I

CHROMATOGRAPHY OF HAEMOGLOBIN ON $Al_2O_3$ UNDER VARYING CONDITIONS

| *$Al_2O_3$ used* | *Pretreatment* | *Hb used* | *Percentage of the different fractions* | | |
|---|---|---|---|---|---|
| | | | *I* | *II* | *III* |
| Merck | — | A | 25 | 52 | 23 |
| BDH | — | A | 35 ± 5 ($n$ = 8) | 46 ± 5 ($n$ = 8) | 19 ± 2 ($n$ = 8) |
| BDH | — | B | 33 | 36 | 31 |
| BDH | — | C | 27 | 52 | 21 |
| BDH | — | F | 31 | 42 | 27 |
| BDH | 96 h dialysis against distilled water | A | 92 | | 8 |
| BDH | 8 h dialysis against 0.35% $NH_3$ | A | 85 | | 15 |
| BDH | 45 h dialysis against 0.35% $NH_3$ | A | 95 | | 5 |
| BDH | 45 h dialysis against 0.35% $NH_3$, recombined with a threefold equivalent amount of the dialysate | A | 79 | | 21 |
| BDH | 45 h dialysis against 0.35% $NH_3$, recombined with a threefold equimolar amount of GSH | A | 81 | | 19 |

while in some experiments two water-mobile fractions were observed. As glutathione (GSH) is the main component in the dialysate, similar experiments were carried out by recombining the dialysed Hb solution with a threefold equimolar amount of GSH. The results are quite similar to those obtained with the concentrated dialysate (Table I).

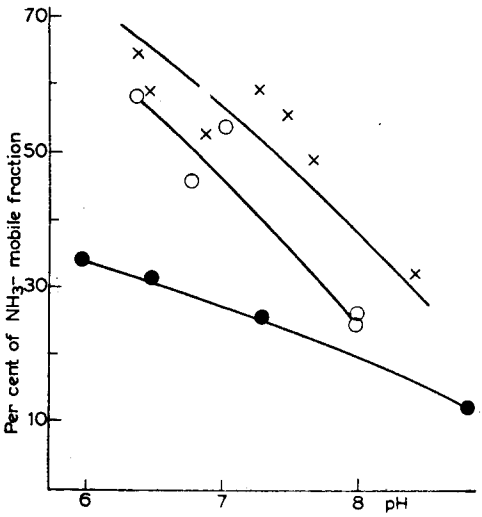


Fig. 1. The influence of the pH of the Hb solution on the percentage of the ammonia-mobile fraction. ●——● without addition; O——O recombined with the concentrated dialysate; ×——× recombined with GSH. In all cases adjustment of the pH with HCl.

From these data it may be concluded that GSH is a prominent factor in the resolution of haemoglobin into three components. However similar results were obtained on examination of haemoglobin solutions with different pH's (obtained by slowly adding very small amounts of hydrochloric acid). The data of Fig. 1 show that a decrease of the pH from 8.8 to 6.0 gave a two- to threefold increase in the amount of the ammonia-mobile fraction. This increase is still greater when concentrated dialysate or GSH was also added (Fig. 1).

Pretreatment of the aluminum oxide with distilled water exerted a great influence on the percentage of the ammonia-mobile fraction. In some experiments small columns (0.9 × 5.0 cm) filled with $Al_2O_3$ were washed with large amounts of distilled water and then used for chromatographic analyses. It was found that the total quantity of haemoglobin remained at the top of the column after a pretreatment with 1500 to 3000 ml of distilled water. In those cases the pH of the eluant was decreased from 9.5 to 7.2 to 6.8.

From these experiments it will be clear that the separation of haemoglobin into three components by aluminium oxide chromatography is dependent on different experimental conditions. It seems very easy to convert one component into the other by slight experimental alterations. The effect of small amounts of hydrochloric acid and of glutathione in increasing the percentage of the ammonia-mobile fraction may be explained by assuming that small amounts of $Al_2O_3$ are converted into $Al(OH)_3$. This aluminium hydroxide is able to absorb large amounts of haemoglobin[10]. The same explanation may be valid for the striking effect of washing the $Al_2O_3$ column with distilled water; it may be that larger amounts of $Al_2O_3$ are converted into $Al(OH)_3$, thus accounting for the high capacity of such a column for absorbing haemoglobin. We believe, therefore, that the apparent heterogeneity of haemoglobin as shown by aluminium oxide chromatography is due to experimental conditions; the use of this method for proving haemoglobin heterogeneity is strictly limited.

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